Dendritic cell-development in steady-state and inflammation

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STATEMENT

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In the summary of the publication and manuscripts I have handed in as my doctoral thesis, I have correctly stated the names of all involved colleagues. I also have correctly stated their contribution in the collaborative work, and I have correctly stated the significance of my part in said work.

Zürich, March 18th 2010

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ABBREVIATIONS

APC	antigen-presenting cell
BM	bone marrow
BMSC	bone marrow stromal cell
cDC	classical dendritic cell
CDP	common dendritic cell progenitor
CFSE	carboxyfluorescein diacetate succinimidyl ester
CFU	colony-formation unit
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CpG	unmethylated CpG-containing oligodeoxynucleotides
DC	dendritic cell
FACS	fluorescence activated cell sorting
Flt3	fms-like tyrosine kinase 3
Flt3L	fms-like tyrosine kinase 3 ligand
G-CSF	granulocyte-colony stimulating factor
GFP	enhanced green fluorescent protein
GM-CSF	granulocyte macrophage-colony-stimulating factor
GM-CSFR	granulocyte macrophage-colony-stimulating factor receptor
GMP	granulocyte/macrophage progenitor
HEV	high endothelial venules
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cells
IFN	interferon
IL	interleukin
IPC	type-I interferon producing cell (equivalent to plasmacytoid dendritic cell)
LC	Langerhans cell
Lin	lineage antigens
LMPP	lymphoid primed multipotent progenitor
LN	lymph node
LPS	lipopolysacchride
LSK	lineage^{-} c-kit^{hi} Sca-1^{+} cells
LT-HSC	long-term hematopoietic stem cell
M-CSF	macrophage-colony-stimulating factor
M-CSFR	macrophage-colony-stimulating factor receptor
MDP	macrophage-DC progenitor
MEP	megakaryocyte/erythrocyte progenitor
MHCcl2	major histocompatibility complex 2
MigDC	migratory dendritic cell
MPP	multipotent progenitor
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PRR	pattern-recognition receptors
RT-PCR	semi-quantitative real-time polymerase chain reaction analysis
SCF	stem cell factor
ST-HSC	short-term hematopoietic stem cell
TLR	Toll-like receptor
TPO	thrombopoietin
T reg	regulatory T cell
WT	wild type

DEUTSCHE ZUSAMMENFASSUNG (Abstract in German)

Die Entwicklung dendritischer Zellen im Grundzustand und während Entzündungen

Dendritische Zellen (DC) sind die maßgeblichen antigenpräsentierenden Zellen und müssen aufgrund ihrer kurzen Lebensdauer permanent durch differenzierende hämatopoetische Stamm- und Vorläuferzellen aus dem Knochenmark ersetzt werden. Welche schrittweise aufeinanderfolgenden Vorläufer für dendritische Zellen existieren und welche Faktoren auf diese Vorläufer im Grundzustand oder während einer Entzündung einwirken, ist bisher nicht im Detail bekannt. Ein für die Entwicklung von dendritischen Zellen im Grundzustand erforderliches Zyotkin ist Flt3L und dendritische Zellen differenzieren sowohl von lymphoiden, als auch von myeloiden Vorläuferzellen, die den entsprechenden Rezeptor Flt3 exprimieren. Darüber hinaus sind die Zytokine GM-CSF und M-CSF für die Entwicklung von dendritischen Zellen von Bedeutung. Dendritische Zellen werden innerhalb der lymphatischen Gewebe in die Hauptgruppen klassische (cDC) und plasmazytoide dendritische Zellen (pDC) eingeteilt. Unsere Forschungsgruppe identifizierte gemeinsamen $Flt3^+$ $M\text{-}CSFR^+$ Vorläuferzellen (CDP) im Knochenmark von Mäusen, von denen einzelne Zellen sowohl cDC als auch pDC *in vitro* generieren können. *In vivo* bilden CDP effizient cDC und pDC im Rückenmark, in der Milz und in den Lymphknoten, sie produzieren jedoch keine anderen Zelltypen. Untersuchungen an Mäusen mit einem Defekt in den Genen für beide Zytokine Flt3L und GM-CSF, haben gezeigt, dass die Kombination beider Zytokine sowohl an der Bildung von DC-Vorläufern, als auch an der Bildung einer bestimmten DC-Untergruppe in der Dermis beteiligt ist. Aufgrund dieser Erkenntnisse schlagen wir ein neues Modell vor, welches besagt, dass zum einen die Zahl der von DC-Vorläufern exprimierten Zytokinrezeptoren und zum anderen die Verfügbarkeit verschiedener Zytokine in der unmittelbaren Umgebung von DC-Vorläufern ihre Entwicklung maßgeblich bestimmen.

Weitere Rezeptoren, sogenannte Toll-like Rezeptoren (TLR), ermöglichen eine direkte Erkennung von Pathogenen während einer Infektion. Wenn Zellen über TLR aktiviert werden, produzieren sie vermehrt entzündungsfördernde Zytokine. Diese beeinflussen indirekt die Blutbildung von Vorläuferzellen im Knochenmark, um

den besonderen Bedürfnissen während einer Infektion optimal gerecht zu werden. Es ist bekannt, dass außer Immunzellen auch nicht-hämatopoetische Stromazellen TLR exprimieren. Unsere eigenen Untersuchungen haben ergeben, dass Stromazellen, die aus dem Knochenmark isoliert wurden, verschiedene TLR exprimieren, insbesondere TLR4. Wir untersuchten chimäre Mäuse, die einen Defekt in TLR4 entweder im hämatopoetischen System, oder im Stroma trugen. Dabei zeigte sich, dass die Expression von TLR4 auf Stromazellen stärker zur Sekretion von G-CSF, zum Anstieg von myeloiden Vorläuferzellen und zur erhöhten Produktion von myeloiden Zellen beitrug, als es TLR4 Expression auf hämatopoetischen Zellen tat. Außerdem produzierten Stromazellen aus dem Knochenmark nach TLR-Stimulation vermehrt M-CSF und GM-CSF, was zur erhöhten Bildung von dendritischen Zellen führen könnte.

Neben reifen Immunzellen und Stromazellen weisen neueste Daten darauf hin, dass Vorläuferzellen selbst TLR exprimieren. Wir untersuchten die Expression von TLR auf den neu identifizierten CDP und wiesen eine relativ hohe Expression von mRNA für *Tlr2*, *Tlr4* und *Tlr9* im Vergleich zu anderen Vorläuferzellen nach. Um die biologische Relevanz ihrer TLR-Expression zu untersuchen, stimulierten wir CDP mit den entsprechenden TLR-Agonisten und fanden starke Effekte in der differenziellen Regulation von Chemokinrezeptoren, welche die Zellwanderung regulieren. Zwei wichtige Chemokinrezeptoren sind CXCR4, das Vorläuferzellen im Knochenmark festhält, und CCR7, das für die Wanderung von dendritischen Zellen und T-Zellen zu den Lymphknoten essentiell ist. Nach nur zwölf Stunden Stimulation mit TLR-Agonisten *in vitro* regulierten CDP CXCR4 herunter und regulierten gleichzeitig CCR7 herauf. Wir injizierten *in vivo* den CXCR4-Antagonisten AMD3100 und fanden eine rasche Mobilisierung von CDP aus dem Knochenmark in den Blutstrom, in die Milz und in die Lymphknoten. Außerdem transplantierten wir CDP und stellten fest, dass CDP achtmal mehr dendritische Zellen in entzündeten Lymphknoten als in Kontrolllymphknoten bildeten. Diese Ergebnisse deuten auf einen bisher unbekannten TLR-abhängigen Mechanismus hin, der die Wanderung von DC-Vorläufern durch eine differentielle Expression von Chemokinrezeptoren reguliert. Dabei führt eine Ausbreitung von Pathogenen zur direkten Stimulation von Vorläuferzellen im Knochenmark, zu ihrer Mobilisierung in den Blutstrom und zu ihrer Rekrutierung in entzündete Lymphknoten. Insgesamt wird so die Zahl von dendritischen Zellen während einer Immunantwort reguliert.

ABSTRACT

Dendritic cells (DC), the major antigen-presenting cells, continuously need to be regenerated from bone marrow (BM) hematopoietic stem and progenitor cells (HSPC). What intermediate progenitors exist on the way to DC generation and what external factors act on these in steady-state and during inflammation, has not been addressed in detail. Flt3L is a non-redundant cytokine in DC development and the generation of DCs was shown to proceed along both $Flt3^+$ common lymphoid and common myeloid progenitors. Two important additional cytokines known to be involved in DC generation are GM-CSF and M-CSF. In lymphoid organs, the two main DC subsets are plasmacytoid (pDC) and classical DCs (cDC). We identified a common $Flt3^+$ $M\text{-}CSFR^+$ DC progenitor (CDP) in the BM of mice, which on a single cell level gave rise to pDC and cDC *in vitro* and *in vivo* efficiently generated pDCs and cDCs in BM, spleen, and lymph nodes (LN), but no other cell types. Generating mice deficient in both Flt3L and GM-CSF revealed that the combined action of these two cytokines is required for the maintenance of DC progenitors and the generation of some DCs in the dermis. Integrating the most recent findings, we propose a refined model, in which the availability of cytokines in microenvironments and the expression of cytokine receptors determines instructive DC lineage commitment from upstream progenitors.

During infectious challenges, cells expressing Toll-like receptors (TLR) can sense the presence of pathogens, secrete inflammatory cytokines, and influence hematopoietic development to meet urgent needs. In addition to cells of the immune system, non-hematopoietic stromal cells express TLRs. We found that stromal cells isolated from the BM express several TLRs, in particular TLR4. Investigating the biological impact of stromal expressed TLR4 *in vivo*, we found that upon stimulation with TLR4 agonists the stroma compartment contributed to a greater extent to the secretion of G-CSF, the increase of myeloid progenitors, and the enhanced production of myeloid cells than hematopoietic cells did. BM stromal cells further increased the secretion of M-CSF and GM-CSF. Besides mature immune cells and stroma components, recent data suggest that progenitor cells themselves express TLRs. We were the first to investigate TLR expression on DC-restricted progenitors and found relative high expression of *Tlr2*, *Tlr4,* and *Tlr9* mRNA by CDPs. Upon TLR-activation *in vitro*, CDPs rapidly down-regulated CXCR4 and upregulated CCR7.

When blocking CXCR4 *in vivo*, CDP were mobilized from the BM and upon adoptive transfer, CDP-derived DCs specifically increased in inflamed LNs. These findings suggest a novel TLR-mediated mechanism regulating DC progenitor migration and recruitment to sites of inflammation via the differential expression of chemokine receptors. This could help to restore sufficient DC numbers in reactive LNs. Together, our data shed light on the regulatory mechanisms that lead to the generation of specific DC subsets from intermediate progenitors. This information may help future research to more specifically modulate immune responses and treat human disease.

INTRODUCTION

Dendritic cells and their migration to lymphoid organs

Dendritic cells (DC) are part of the hematopoietic system and are characterized by their highly efficient antigen presentation to T cells in lymphoid tissues[1]. DCs are present in primary lymphoid organs, e.g. the bone marrow (BM) and thymus, where they present self-antigens and help to select lymphocytes that carry non-self reactive immune receptors from a randomly generated pool[2]. These naïve lymphocytes circulate through the body and may detect molecular details of invading microbial pathogens. In secondary lymphoid organs, such as spleen and lymph nodes (LN), DCs are involved in the maintenance of peripheral tolerance, and upon infectious challenge efficiently present antigens derived from the pathogen and induce a specific immune response[1]. The two main subsets of DCs present in lymphoid organs are classical DCs (cDC) and plasmacytoid DCs (pDC, also called natural type-I interferon producing cells or IPCs).

To detect a microbial infection, DCs are uniquely equipped with a limited set of innate, germ line-encoded pattern-recognition receptors (PRR), which sense highly conserved structures of pathogens, so-called ‘pathogen-associated molecular patterns’ (PAMP)[3]. In particular, Toll-like receptors (TLR) are located within cellular membranes at the surface or in endosomal vesicles to detect extracellular pathogens[4]. cDCs show typical dendritic extensions, take up and present antigens[1]. Upon activation via TLRs, cDCs upregulate major histocompatibility complexes (MHC) and co-stimulatory molecules to efficiently present pathogen-derived antigens

and prime antigen-specific naïve T cells. Depending on the pathogen's nature, DCs secrete particular cytokines and shape the adaptive response by inducing defined effector and memory T cell subsets[5]. pDCs have a spherical shape, are found in BM, spleen, LN, and liver and are preferentially recruited to sites of inflammation[6]. Upon activation, in particular during viral infections, pDCs produce large amounts of type-I interferons (IFN), develop a cDC-like morphology[7], and to some extend present viral antigens[8].

In addition to lymphoid organs, DCs exist in non-lymphoid tissues, which can be subdivided into sterile tissues, such as the pancreas and heart, in filtering sites, such as the liver and kidney, and in environmental surfaces, such as the lung, gut, and skin[9]. In steady-state, low numbers of these 'non-lymphoid tissue DCs' continuously migrate to the draining LNs via afferent lymphatics and are then referred to as 'migratory DCs' (MigDC)[10-12]. This migration depends on signals by the chemokine receptor CCR7[13-14] and its two ligands, CCL21 and CCL19, which are expressed on HEVs, by stromal cells in the T cell zones, and by lymphatic endothelial cells[15]. Upon inflammation, the numbers of DCs entering the LN via afferent lymphatics increase due to upregulation of CCL21 (SLC) on lymphatic endothelial cells[16]. This DC migration is further amplified by the upregulation of CCR7 on activated DCs[17-20].

In a second CCR7-dependent route, DCs are expected to enter LNs from the blood via high endothelial venules (HEV)[13, 15, 21-22]. The transmigration through HEVs depends on CD62L-mediated rolling on the inner wall of the vessels, the activation of chemokine receptors such as CCR7, and subsequent integrin-mediated firm adhesion[23]. The evidence that DCs, like $CD8\alpha^+$ cDC enter the LN via HEV is however indirect, and has been convincingly shown only for pDCs[11]. This suggests that in steady-state DC progenitors may enter LNs and locally give rise to LN resident cDCs (see manuscript Schmid *et al*. "TLRs on common dendritic progenitors" in the appendix).

Several other chemokine receptors were suggested to play a role in DC migration into lymphoid organs, particularly under inflammatory conditions: CCR2 was shown to mediate LN entry of monocytes and inflammatory monocyte-derived DCs[24]; CXCR3 and CCR5 were shown to play a role in pDC entry into inflamed LNs[25-27]; and CX_3CR1 plays a role in monocyte entry into the spleen[28], but is probably not involved in DC development and a role in DC migration yet remains to

be investigated. Interestingly, a recent study further suggested that DC migration to the LN may directly be linked to antigen presentation via the MHCcl2-associated invariant chain 'Ii' (CD74)[29].

Cytokines in DC development

DCs have a short half-life of 4 days to maximal 2 weeks in lymphoid organs and continuously need to be replenished from BM hematopoietic stem and progenitor cells (HSPC)[30-33]. Granulocyte macrophage-colony stimulating factor (GM-CSF) was the first cytokine discovered to efficiently drive DC differentiation from monocyte or cultures of BM cells *in vitro*[34-36]. However, these DCs were later discovered to be morphologically and functionally distinct from *ex vivo*-isolated cDCs[37], and are now considered to correlate with inflammatory DCs that are derived from monocyte precursors (see manuscript Schmid *et al*. Immunol Rev (2010) 234, in the appendix for more details). *In vivo* GM-CSF is induced upon inflammatory stimuli by stromal cells such as endothelial cells and fibroblasts, as well as by hematopoietic cells like macrophages, mast cells, osteoblasts, and activated T and B cells[38]. Data obtained from research in mice further support the view that GM-CSF primarily acts during inflammation, but is less relevant for steady-state DCs: Neither GM-CSF deficient mice, mice lacking the GM-CSFR common β chain, nor transgenic mice over-expressing GM-CSF showed major changes in cDC numbers; and adoptively transferred monocytes generated non-lymphoid tissue DCs and inflammatory DCs in the spleen, but no cDCs in steady-state[37-41].

Similar to GM-CSF, addition of Flt3-ligand (Flt3L) to *in vitro* cultures of BM cells results in the generation of DCs[42]. In contrast to GM-CSF, Flt3L-driven cultures produce cDCs and pDCs from BM progenitors, but not from monocytes[37, 43]. *In vivo*, stromal cells, endothelial cells and activated T cells are the main sources of Flt3L, and to lesser extend B cells, and myeloid cells[44-46]. Mice deficient in Flt3L have severely reduced cDCs and pDCs[47], and the repetitive injection or conditional expression of Flt3L leads to the massive expansion of cDCs, pDCs, and myeloid cells but not of B or T lymphocytes[48-50]. These findings suggest a non-redundant role of Flt3L in steady-state DC development.

In addition to Flt3L and GM-CSF, initial studies have demonstrated that macrophage-colony stimulating factor (M-CSF) and its receptor, M-CSFR (CD115) contribute to DC development: Mice lacking M-CSF ($Csf1^{op/op}$ mice) have a two to

three-fold reduction in spleen cDCs and pDCs[51], and M-CSF signaling was found to be important for the generation of Langerhans cells (LC)[52], which is the subset of non-lymphoid tissue DCs present in the epidermis. *In vivo*, M-CSF is produced by stromal cells, endothelial cells, macrophages, and osteoblasts[53]. However, the relative contribution of these three cytokines to the generation of functionally distinct DC subsets has not been clarified in detail.

Dendritic cell progenitors

The differentiation of hematopoietic stem cells (HSC) to mature cells is characterized by the progressive loss of developmental options and the generation of intermediate progenitors restricted to certain lineages[54]. These include on the one hand common myeloid progenitors (CMP), which further give rise to granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythrocyte progenitors (MEP)[55]; and on the other hand, common lymphoid progenitors (CLP)[56] give rise to all cells from the lymphoid lineage, including B cells, T cells, and NK cells. Despite this classical segregation of hematopoietic differentiation into a myeloid and a lymphoid branch, DC development was shown to occur from both myeloid as well as lymphoid progenitors[30-31, 57-58]. This represents a unique redundancy in hematopoietic development, which until now has not been observed for any other lineage. The biological relevance this may have has not yet been discussed in detail.

Several intermediate DC progenitor populations have been identified in mice. Since pDCs and cDCs have partly overlapping functions and their generation requires similar cytokines, they were expected to develop from a common progenitor. Initially, del Hoyo *et al*. suggested a $CD11c^+$ $MHCII^-$ population in peripheral blood to contain common dendritic progenitors, but discovered later that the isolated population had contained a heterogeneous mix of different progenitors and NK cells[59-60]. One further report described that Lin^- $c\text{-}Kit^+$ CX_3CR1^+ single cells isolated from the BM of Cx3cr1-GFP reporter mice differentiated to monocytes, macrophages, and cDCs[61], but not to pDC. At that time, it remained unclear, how the identified common macrophage and DC progenitor (MDP) could be integrated into the hypothesis of a common developmental route for pDCs and cDCs. The only DC-restricted progenitors that had been characterized were $CD11c^+$ $CD45RA^{lo}$ $CD43^{int}$ SIRP-α^{int} $MHCcl2^-$ ‘pre-cDCs’ in the spleen of mice, which generated all cDC subsets but no pDCs, monocytes or macrophages[40]. In the following, it is described how we

characterized a common dendritic progenitor (CDP) giving rise to pDCs and cDCs, but no other lineages.

The identification of DC progenitors in the BM and DC-restricted pre-cDCs in the spleen further raised the question of which intermediate progenitor would exit the BM and seed into the periphery. The chemokine receptor CXCR4 was demonstrated to be a key regulator for human and mouse HSPC migration, their retention in the BM and re-homing upon circulation through the blood[62-64]. AMD3100 is an antagonist that blocks the interaction of CXCR4 with its cognate ligand CXCL12 and leads to the transient mobilization of HSPCs from the BM into blood circulation[65-66]. CXCR4 was further shown to be essential for pDC development within CXCL12-rich niches in the BM microenvironment[67].

The regulation of hematopoiesis during inflammation

It has long been observed that during severe infections, like bacterial sepsis or systemic viremia, hematopoietic development is skewed toward lineages of urgent need[68-70]. Non-hematopoietic stromal cells and mature cells of the hematopoietic system were both shown to express TLRs and upon activation produce inflammatory cytokines[71-72]. These distribute via the bloodstream, reach to BM, and indirectly act on HSPCs to regulate their hematopoiesis during these emergency conditions. Nevertheless, how much the stroma compartment and hematopoietic cells each contribute to the production of inflammatory cytokines has not been addressed in detail. We hypothesized that in particular stromal cells in the BM may express TLRs and influence HSPCs locally, once PAMPs from a severe infection spread to the highly vasculated BM (see manuscript Ziegler *et al*. ‘BMSCs support myelopoiesis upon demand’ in the appendix).

Interestingly, there is one further class of cells that may contribute to the detection of PAMPs in the BM and influence hematopoiesis during an ongoing infection: the hematopoietic progenitors themselves. Recent findings have shown that HSPC express TLRs and could directly sense PAMPs[73-79]. Results from these studies suggest that the activation via TLRs directly induces the differentiation of HSPCs to myeloid cells and DCs or influences the migration of circulating HSCs. However, no information about TLR expression on DC-restricted progenitors is currently available.

In the work presented here, we set out to define intermediate DC-restricted progenitors; further characterize the cytokines and local environments, which instruct DC lineage commitment in steady-state and during inflammation; and in particular focused on investigating the biological relevance of TLR expression by DC-restricted progenitors to regulate the replenishment of DC subsets in inflamed lymphoid organs upon microbial challenge (see manuscript Schmid *et al*. "TLRs on common dendritic progenitors" in the appendix).

RESULTS

The role of Flt3 in the search for a common dendritic progenitor

To discover the successive intermediate DC progenitors allows future research to establish the mechanisms that regulate DC generation in health and disease. We set out to identify a common DC-restricted progenitor for pDCs and cDCs using a rational approach to dissect progenitor fractions in the BM by their cytokine receptor expression. As discussed above, Flt3L is a major, non-redundant cytokine for DC development[80-82]. The receptor for Flt3L, fms-like tyrosine kinase 3 (Flt3, also known as Flk2), a type-III receptor tyrosine kinase, is continuously expressed from progenitor cells to steady-state DCs[82-83]. In line with this, only the $Flt3^+$ fractions of both myeloid and lymphoid progenitors are capable of giving rise to DCs[83], and the ectopic expression of Flt3 enhances DC potential in $Flt3^+$ progenitors, and induces $Flt3^-$ progenitors to give rise to DCs[84]. Consequently, we and others have concluded that DC development proceeds along a successive line of $Flt3^+$ progenitors and is driven by Flt3L[83, 85-86]. Based on these findings, we proposed a ‘Flt3 license’ working model for pDC and cDC differentiation, in which continuous strong Flt3L signals lead to DC development from upstream $Flt3^+$ progenitors to $Flt3^+$ steady-state DCs, whereas competing signals may lead to alternative lineage outcomes (see Figure 1, Onai *et al*. Ann N Y Acad Sci (2007) in the appendix). We concluded that a common dendritic progenitor would most likely express Flt3.

Flt3$^+$ M-CSFR$^+$ common dendritic progenitors (CDP) give rise to plasmacytoid and classical dendritic cells in steady-state lymphoid organs

The other candidate cytokine receptors likely expressed by a common dendritic progenitor were GM-CSFR and M-CSFR. As we were not able to obtain an antibody that could consistently detect mouse GM-CSFR, we focused on Flt3 and M-CSFR. Within the lineage negative (Lin$^-$) fraction of mouse BM cells (*i.e.* cells not expressing surface markers of mature hematopoietic lineages), those expressing high levels of c-Kit, the receptor for stem cell factor (SCF), contain HSCs, multipotent progenitors (MPP), and myeloid progenitors, whereas cells expressing intermediate or low levels of c-Kit contain IL7Rα^+ lymphoid and other more committed progenitors. Common DC-restricted progenitors would therefore likely be contained within the Lin$^-$ c-Kit^{int} Flt3$^+$ fraction. We identified a population of Lin$^-$ c-Kit^{int} Flt3$^+$ M-CSFR$^+$ IL7Rα^- cells that accounted to approximately 0.1% of BM nucleated cells[87]. N. Onai and A. Obata-Onai initially characterized this population to give rise to pDCs and cDCs on a single-cell level *in vitro*. They further performed *in vivo* adoptive transfer assays into irradiated hosts and found pDC and cDC-offspring in BM and spleen, but no cells of other lineages under most permissive conditions *in vivo* and *in vitro*. We therefore termed them "common dendritic progenitors (CDP)". Naik *et al*.[88] in parallel identified a similar pDC and cDC-restricted progenitor in an *in vitro* culture system, confirming and extending our *in vivo* findings of a DC-restricted clonogenic common progenitor. The initial aims of the presented work were to further characterize CDPs and their offspring *in vivo* during steady-state, establish their relative position downstream of common myeloid and lymphoid progenitors, and optimize their isolation procedure. We found that CDPs showed typical progenitor morphology and actively proliferated in steady-state BM, with approximately 38 % of cells in S+G_2+M phase of the cell cycle. By establishing adoptive transfers of CDPs into non-conditioned steady-state hosts, we confirmed engraftment in BM and spleen from transfers into irradiated hosts, and were the first to demonstrate that BM progenitors give rise to pDCs and cDCs in LNs. To determine the relative position of CDPs to other BM progenitors, HSCs, myeloid or lymphoid progenitors were transferred into the BM cavity of the tibia of mice. On day four, upstream Lin$^-$ c-Kit^{hi} Sca-1$^+$ MPP and HSC, as well as Flt3$^+$ myeloid progenitors gave rise to CDPs by phenotype, but no CDP offspring from Flt3$^+$ lymphoid progenitors was detected. This suggests that DC differentiation along the myeloid branch of hematopoiesis proceeds via CDPs. If DCs from lymphoid

progenitors also develop via CDP as an intermediate state or follow an alternative developmental path remains elusive to date (see Onai *et al*. Nat Immunol (2007) in the appendix).

To integrate our findings into future research, it was essential to establish an efficient and reproducible way to isolate CDPs from the BM. Therefore, we established a new pre-selection method for BM progenitors by indirect immuno-magnetical labeling and depletion of mature Lin^+ cells (see Onai *et al.* Methods Mol Biol (2010) in the appendix). Since we and others[81] could not at first reproduce the key finding that Lin^- M-$CSFR^+$ progenitors produced cDCs as well as substantial numbers of pDCs, but rather mostly cDC offspring was detected, it became necessary to further refine the different BM progenitor sub-fractions. By optimizing the resolution of M-CSFR detection during the fluorescence activated cell sorting (FACS), we separated Lin^- c-Kit^{int} $Flt3^+$ BM progenitors into M-CSFR high expressing, low positive, and negative fractions. This revealed that M-CSFR high expressing cells are committed to cDCs, whereas 'true CDPs', carrying bi-potential to produce pDCs as well as cDC, are M-CSFR low positive (see manuscript Schmid *et al*. Immunol Rev (2010) 234 in the appendix). This refined method of isolating CDPs was communicated during the 10^{th} International Symposium on Dendritic cells 'DC 2008' poster presentation in Kobe, Japan and was published as a practical manual (see Onai *et al.* Methods Mol Biol (2010) in the appendix).

The combined action of cytokines maintains DC progenitor numbers in the bone marrow and their offspring in the periphery

We next determined the contribution of different cytokines on pDC and cDC generation from CDPs. *In vitro*, CDPs had given rise to pDCs and cDCs in Flt3L supplemented cultures and to only cDCs when cultured in presence of GM-CSF. When we *in vivo* injected Flt3L into steady-state non-conditioned recipients, we found a substantial increase of CDP-derived pDCs and cDCs in LNs and spleen on day 8. As M-CSF was further suggested to contribute to DC generation and CDPs are isolated via their expression of M-CSFR, we added M-CSF to cultures of CDPs and found that M-CSF strongly increased pDC offspring and to some extend cDCs (see Onai *et al*. Nat Immunol (2007) in the appendix). A subsequent study confirmed this finding and further suggested that both pDC and cDC-generation is supported by M-CSF even in the absence of Flt3L[89].

It was next essential to investigate the combined action of different cytokines on the commitment of upstream progenitors towards the DC lineage. Thus we determined the numbers of CDPs and MDPs in the BM of mice deficient in either Flt3L or GM-CSF and of newly generated double deficient animals. We found that single deficient animals had somewhat reduced numbers of both progenitor fractions compared to wild type (WT), but double deficient animals showed a significant further decrease. Flt3L deficient mice had previously been shown to have a 10-fold reduction of pDCs and cDCs in steady-state BM and spleen[80]. However, the additional absence of GM-CSF in double deficient animals did not lead to a further reduction of pDCs and cDC, even though DC progenitors were reduced (see Kingston *et al*. Blood (2009) in the appendix). This suggests additional compensatory effects in place to counteract the reduced number of DC progenitors on their way to give rise to pDCs and cDCs in steady-state lymphoid organs. Nevertheless, the $CD11b^+$ subset of dermal DCs and in line with this, MigDCs in skin-draining LNs, were reduced in the GM-CSF / Flt3L double deficient compared to single deficient animals. In accordance with this, they were less efficient in priming naïve T and B cell responses during the initial phase of the immune response. These findings support the importance of Flt3L as a cytokine driving steady-state DC generation from $Flt3^+$ progenitors. They further establish a new role of M-CSF and emphasize the role of GM-CSF in combination with Flt3L in the maintenance of DC progenitors and generation of some non-lymphoid tissue DCs.

Stromal cells produce inflammatory cytokines upon TLR activation and support hematopoiesis during systemic inflammation

Having gained further insight into DC progenitors and their cytokine requirements in steady-state, we proceeded to study hematopoiesis and DC development during inflammation. To follow this aim, we first studied the relative contribution of the hematopoietic and the non-hematopoietic stroma compartment to the production of inflammatory cytokines. Hematopoietic immune cells are specialized to fend off microbial infections, express TLRs, and upon activation secrete inflammatory cytokines[72]. More recently, non-hematopoietic stroma components, such as intestinal epithelium or endothelial cells in the lung, were shown to express TLRs and contribute to the inflammatory response[71, 90]. We set out to further study the

mechanisms involved and in particular focus on stromal cells in the BM, where myelopoiesis primarily takes place.

When isolated by plastic adherence, we found that human BM stromal cells (BMSC) expressed several TLRs, in particular TLR4. Upon stimulation with lipopolysaccharide (LPS) and other PAMPs *in vitro*, they increased the secretion of G-CSF, M-CSF, and GM-CSF and supported an enhanced maintenance of human early hematopoietic progenitors (see manuscript Ziegler *et al*. 'BMSCs support myelopoiesis upon demand' in the appendix). To verify these effects *in vivo*, we generated chimeric animals, in which either the hematopoietic or the non-hematopoietic compartment was deficient in TLR4 signaling. We found that intact TLR4 signaling within the stroma compartment contributed more to an increase in myeloid progenitors in the BM, HSPCs in the blood, and myeloid cells in the spleen than hematopoietic cells did. We detected an increased production of G-CSF by stromal cells in the BM, spleen, and lung. To check the relevance of these findings on DC development, we sought to analyze the numbers of CDPs and other DC progenitors in the chimeric animals. However, the injection of LPS made it impossible to detect Flt3 or M-CSFR expressing cells in the BM, independent of whether TLR4 signaling within the hematopoietic or the stroma compartment was intact (data not shown). This may have been due to increased levels of M-CSF and of Flt3L (data in WT animals, not shown), which prevented surface detection of the receptors by internalization or by blocking antibody binding.

Direct sensing of TLR agonists by CDPs leads to CXCR4 down-regulation, CCR7 upregulation and increased numbers of DCs in inflamed lymph nodes

Some initial reports demonstrated that TLR stimulation of HSPC induced DC generation[75, 77, 79] or influenced HSPC migration[78]. However, no information about TLR expression on DC-restricted progenitors was available at that time. We demonstrated that CDPs express higher levels of *Tlr2* and *Tlr9* mRNA than other BM progenitors[77, 79] and intermediate levels of *Tlr4*. We hypothesized that TLR activation of CDPs may influence their migratory behavior. In steady-state, CDPs expressed relatively high levels for CXCR4, CD34, and CD44, but were low or negative for CCR7, CXCR3 or CD11c. Similar profiles of migratory markers are typical for BM-resident progenitors, but CDP further expressed high level of CD62L, known to be involved in rolling on HEVs and entry into LNs. Upon direct TLR activation,

CDPs rapidly down-regulated CXCR4 and upregulated CCR7 (see manuscript Schmid *et al*. "TLRs on common dendritic progenitors" in the appendix).

To test whether CXCR4 down-regulation may lead to the mobilization of CDPs from the BM, we injected the CXCR4 antagonists AMD3100 and found that CDPs were mobilized from the BM and entered the spleen and LNs. This suggests that CDP localization in the BM does depend on CXCR4 and once mobilized, CDP have the machinery to enter LNs. To test whether CDPs are mobilized in an inflammatory setting, we intravenously injected PAMPs. Only 12 h later, no Lin^- cells expressing Flt3 or M-CSFR, including CDPs, were detectable in BM, blood or spleen. Similarly as described above, this likely resulted from an increased production of the cytokines Flt3L and M-CSF and ceased detection of the respective cytokine receptors. To further characterize the functional relevance of TLR expression *in vivo*, we transferred CDPs into mice and injected CpG into the front foot pad. On day 4, we determined an 8.8-fold increase of CDP-derived plasmacytoid DC, classical DC, and MigDC in inflamed, compared to control LNs, but no increased local proliferation. This indicates that CDPs, or their offspring, are preferentially recruited to inflamed LNs. These findings suggest a novel TLR-mediated mechanism that regulates DC progenitor migration during inflammation via the differential expression of chemokine receptors.

DISCUSSION

The revised tree for dendritic cell development

The identification of CDPs as a common progenitor for pDC and cDC-generation drove major progress in the understanding of DC development. To allow other investigators to incorporate our finding into their own research, it was essential to establish a reproducible isolation protocol for CDPs. We could contribute to this by providing an improved pre-selection procedure and a refined surface phenotype of CDPs as Lin^- c-Kit^{int} $Flt3^+$ IL7Rα^- M-$CSFR^{low+}$ for cell sorting. CDPs are currently being incorporated into an emerging new picture of DC development.

When we isolated CDPs, the two parallel findings that MDPs gave rise to monocytes, macrophages, and cDCs[61] and that CDPs produced cDCs, as well as pDCs[87], was difficult to interpret. This issue was resolved when MDPs were

subsequently found to also give rise to pDCs[28, 91]. It became clear that MDPs are the immediate progenitors of CDPs[92] and give rise to CDPs when the monocyte/macrophage lineage separates from the DC lineage. cDC-generation from CDPs was demonstrated to occur via pre-cDC[92]. CDPs were also shown to be capable of generating some DC subsets in non-lymphoid tissues, such as the gut, liver, and kidney[93-95], which further extends their *in vivo*-relevance. The generation of non-lymphoid tissue DCs from CDPs also occurs via pre-cDCs[40], making it more appropriate to term them 'pre-DCs'. We summarized the most recent findings in an integrative view of DC development, which will be published in the March 2010 issue of Immunological Reviews (see Figure 1 of the manuscript Schmid *et al*. Immunol Rev 234 in the appendix).

Instructive cytokine signals in dendritic cell lineage commitment

Our detailed analysis about the cytokine requirement of upstream progenitors to commit to the DC lineage and subsequently generate sufficient numbers of DCs in lymphoid and non-lymphoid tissues[82, 87] helped to better understand the extrinsic signals that regulate DC development. Ginhoux *et al*. further built on these results to characterize the cytokine requirements of different DC subsets in several non-lymphoid tissues[95]. When measuring cytokine receptor expression on successive progenitors during DC differentiation, cytokine receptor expression closely correlated with lineage commitment. We developed a new concept where the set of expressed cytokine receptors determine the lineage potential of an intermediate progenitor by enabling it to receive instructive signals and differentiate to the corresponding lineage (see Figure 3 of the manuscript Schmid *et al*. Immunol Rev 234 in the appendix). Consequently, lineage differentiation of BM progenitors seems to be determined by their cytokine receptor expression and the availability of different combinations of cytokines within defined microenvironments. In case of DC development, the balanced action of Flt3L, M-CSF, and GM-CSF should determine whether MDPs give rise to macrophages or via monocytes to inflammatory DCs or whether they commit to the DC lineage by generating CDPs and subsequently give rise to pDCs, cDCs, and $CD11b^+$ non-lymphoid tissue DCs. For a more detailed illustration of these concepts, please see Figure 2 of the manuscript Schmid *et al*. Immunol Rev 234 'Instructive cytokine signals in dendritic cell lineage commitment' in the appendix.

The relative contribution of TLR expression by stromal cells and by DC progenitors on the sufficient supply of DCs during infectious challenges

As we describe above, cytokines play a major role in DC development. The main cytokines in steady-state, Flt3L and M-CSF, are constitutively produced to maintain DC homeostasis. However, GM-CSF is only detectable upon inflammation. It has long been discussed that stroma cells support hematopoiesis during severe systemic inflammation. We set out to determine the relative contribution of stromal cells, particularly in the BM, to locally support myeloid cell generation and refine some of the underlying mechanisms. We could show that human BMSC expressed several TLRs, in particular TLR4, and produced increased amounts of G-CSF, GM-CSF, and M-CSF upon stimulation *in vitro*. In chimeric mice the total stroma compartment contributed more to the production of G-CSF, increased myeloid progenitors in the BM, and raised numbers of granulocytes in the spleen than the hematopoietic system did. Although WT mice increased the expression of G-CSF mRNA in the BM, it also increased in the spleen and the lung, making the *in vivo* contribution of stromal cells in the BM, compared to other tissues, difficult to judge. Nevertheless, since hematopoiesis mostly takes place within the BM it seems likely that cytokines produced by BM stromal cells locally play a major role. Human BMSC are already implicated in the immune modulation of several clinical studies, although they have not yet adequately been characterized. We here attempted to bridge *in vitro* studies on human BMSCs with *in vivo* studies on chimeric mice. We discovered striking parallels in cytokine production and the maintenance of progenitors in both systems, which make it possible to conclude that there actually is a major contribution of stromal cells to enhance myelopoiesis during LPS-induced inflammation and that similar effects may be true for DC generation. Future studies will show whether different subsets of human stromal cells in the BM may contribute to modulate immune responses and upon deeper understanding of their biology, these may successfully be applied to treat human disease.

In contrast to TLR expression by stromal cells and indirect regulation of hematopoiesis via cytokines, we further showed that CDPs express TLRs and can directly sense products of microbial pathogens. Direct TLR stimulation of CDPs led to the rapid down-regulation of CXCR4 and upregulation of CCR7. *In vivo*, blocking CXCR4 led to the mobilization of CDPs from the BM. It is suggestive that a down-regulation of CXCR4 would similarly mobilize CDPs from the BM upon TLR triggering

in vivo, and we are currently addressing this issue in more detail. We followed the hypothesis that upregulation of CCR7 may lead to a preferential recruitment of CDPs to inflamed LNs. CDPs transferred into locally CpG-injected animals gave rise to 8.8-fold higher numbers of DCs in inflamed LNs, than in control LNs; however, there was no increase in local proliferation. This suggests that upregulation of CCR7 may indeed lead to the direct recruitment of CDPs or enhanced recruitment and/or survival of their DC offspring. To further address these issues, we are currently transferring CCR7-deficient and WT CDPs in competition to test whether enhanced numbers of DCs in inflamed LNs indeed depend on CCR7 upregulation upon TLR triggering. Together, this study confirms the view that CDPs in steady-state predominantly reside within the BM, whereas pre-DCs are the main circulating DC-restricted progenitors that leave the BM and seed peripheral lymphoid organs[92-93, 95]. However, the mobilization of CDPs from the BM upon TLR triggering and recruitment to reactive LNs may enable the generation of higher numbers of DCs due to their high proliferative capacity[87] and the local production of pDCs upon specific demand. These effects may help to maintain sufficient DC numbers in reactive LNs during late phases of the immune response and to restore DC homeostasis once the inflammation ceases. Furthermore, synergistic effects could occur when several TLRs on stromal or progenitor cells are triggered in parallel and different classes of bacteria or viruses may lead to different types of responses, depending on the specific set of TLRs that are activated.

Conclusion

We can conclude that the identification of intermediate progenitors during DC generation has led to a substantial increase in our understanding of how different progenitors from the BM can seed different lymphoid and non-lymphoid tissues to locally give rise to specific subsets of DC. The differentiation of various DC subsets seems to predominantly be regulated by specific combinations of cytokines present in local microenvironments. Upon infectious challenges, TLR triggering of stromal cells and immune cells increases the production of cytokines, which act on DC progenitors and determine the differentiation of functionally distinct and more abundant numbers of DCs. The migratory behavior also strongly differs in steady-state and upon acute infection and may be regulated by directly triggering of TLRs expressed by DC-restricted progenitors. As underlying concepts in DC development

were shown to be conserved in human and mice, we believe that the recent advances in the field will help to modulate immune responses and ultimately improve the treatment of human disease.

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LIST OF MANUSCRIPTS & PUBLICATIONS – AUTHORS CONTRIBUTION

1. Schmid MA, Kingston D, and Manz MG.
   Direct sensing of Toll-like receptor agonists by common dendritic progenitors leads to CXCR4 down-regulation, CCR7 upregulation and increased numbers of dendritic cells in inflamed lymph nodes.
   Manuscript.

   M.A.S. designed and performed the experiments for all figures, analyzed and interpreted data, and wrote the manuscript. D.K. helped performing experiments, collected and discussed data, and edited the manuscript. M.G.M. directed the study, discussed data and edited the manuscript.

2. Schmid MA, Kingston D, Boddupalli S, and Manz MG.
   Instructive cytokine signals in dendritic cell lineage commitment.
   Immunol Rev (2010) 234(1):32-44.

   M.A.S. wrote the manuscript. DK helped writing the manuscript and designed figures. S.B. editing the manuscript. M.G.M. directed the work, discussed concepts, and edited the manuscript.

3. Ziegler P, Boettcher S, Schmid MA, Garavaglia G, Takizawa H, and Manz MG.
   Bone marrow stromal cells sense TLR4 agonists and subsequently enhance myelopoiesis. Manuscript.

   P.Z. and S.B. contributed equally to the work, performed experiments, collected data, discussed results, and helped writing the manuscript. M.A.S. helped to generate the chimeric animals (Fig. 3, 4, 5, and Suppl. Fig. S4), performed experiments and acquired the data for Figure 4, Figure 5 A and C, and Supplementary Figure S4. G.G. and H.T. performed further experiments. M.G.M. directed the work, discussed data and wrote the manuscript.

4. Kingston D, Schmid MA, Onai N, Obata-Onai A, Baumjohann D, and Manz MG.
   The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis.
   Blood (2009) 114(4):835-43.

   D.K. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. M.A.S. helped perform experiments, did flow cytometric analysis for Figure 2 and Supplementary Figure S1, and discussed results. N.O., A.O.-O., and D.B. performed further experiments and collected data; and M.G.M. directed the study and co-wrote the manuscript.

5. Onai N, Manz MG, and Schmid MA.
   Isolation of common dendritic cell progenitors (CDP) from mouse bone marrow.
   Methods Mol Biol (2010) 595:195-203, 'Dendritic Cell Protocols' book chapter.

   N.O. had initially established the isolation of CDPs, performed experiments and wrote the manuscript. M.G.M discussed results, and edited the manuscript. M.A.S. optimized the isolation procedure of CDPs by pre-enriching Lin^- cells, revised the gating strategy and surface phenotype of CDPs to be intermediate to low for M-CSFR expression for cell sorting, generated data for Figure 13.1., and co-wrote the manuscript.

6. Onai N, Obata-Onai A, Schmid MA, Ohteki T, Jarrossay D, and Manz MG.
   Identification of clonogenic common Flt3+ M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow.
   Nat Immunol (2007) 8(11):1207-16.

   N.O. and A.O.-O. contributed equally to this work, initially discovered CDPs, and characterized them *in vitro* and *in vivo* in irradiated animals, performed clonal assays, and contributed to the writing of the manuscript. M.A.S. continued the study, revised the cell sorting strategy (Fig. 1 a, b); determined the appearance and cell cycle status of CDPs *ex vivo* (Fig. 1 c, e); confirmed M-CSF stimulated cultures (Fig. 4, and Suppl. Fig. S2); established steady-state adoptive transfers into non-conditioned recipients, and Flt3L injections (Suppl. Fig. S5); investigated the origin of CDPs from upstream progenitors (Suppl. Fig. S8); and contributed to

the writing of the manuscript. T.O. provided advice. D.J. did cell sorting. M.G.M. directed the study, discussed the results, and wrote the manuscript.

7. Onai N, Obata-Onai A, Schmid MA, and Manz MG.
   Flt3 in regulation of type-I interferon producing and dendritic cell development.
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   N.O., A.O.-O., and M.A.S. helped writing the manuscript. M.G.M. directed the study, developed the concepts, and wrote the manuscript.

Appendix:
Manuscripts & publications

Manuscript Schmid *et al*. 'TLRs on common dendritic progenitors'

Direct sensing of Toll-like receptor agonists by common dendritic progenitors leads to CXCR4 down-regulation, CCR7 upregulation and increased numbers of dendritic cells in inflamed lymph nodes

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ABSTRACT

Dendritic cells (DC), the major antigen-presenting cells, continuously need to be regenerated from bone marrow (BM) hematopoietic progenitor cells. During bacterial and viral infections, recent studies suggest that stem and progenitor cells can directly sense microbial products via Toll-like receptors (TLR). We investigated TLR expression on DC-restricted progenitors and found relative high expression of *Tlr2*, *Tlr4* and *Tlr9* mRNA by common dendritic progenitors (CDP). Direct activation of TLRs led to rapid down-regulation of *Cxcr4* and upregulation of *Ccr7* of CDPs *in vitro*. When blocking CXCR4-CXCL12 interaction *in vivo*, CDPs were mobilized from the BM and entered spleen and lymph nodes (LN). Four days after adoptive transfer and CpG-injection into the foot pad, CDP-derived DCs increased 8.8-fold in inflamed, compared to control LNs, but no enhanced local proliferation was observed. Our data suggest a novel TLR-mediated mechanism that may lead to the mobilization of CDPs from the BM and preferential recruitment of DC progenitors and their offspring to reactive LNs. This distinct layer of regulation may help to supply DC subsets required during the ongoing immune response or to restore DCs to return to steady-state conditions.

INTRODUCTION

Hematopoietic stem and progenitor cells (HSPC), residing in the bone marrow (BM), give rise to all lineages of blood cells in steady-state[1]. Dendritic cells (DC) are part of the hematopoietic system, and have specifically evolved to present antigens and link the innate with the adaptive immune response. Most DC subsets have a short half life and therefore continuously need to be replenished from HSPC[2-3]. During infectious challenges, they strongly increase within lymphoid organs[4]. The key events that regulate DC development during inflammation are not understood in detail.

To sense an infection with invading microbial pathogens, a limited set of innate, germline-encoded pattern-recognition receptors (PRR) has evolved. These detect highly conserved microbial structures, so-called pathogen-associated molecular patterns (PAMPs)[5]. One group of PRRs, including Toll-like receptors (TLR), are transmembrane proteins located in the plasma membrane and endosomal/phagocytic vesicles of immune and stroma cells and detect extracellular

pathogens[6]. Other PRRs are cytoplasmic proteins[7], which sense intracellular pathogens and the damage within infected cells[8]. The innate detection of pathogens induces an inflammatory response, which shapes the quality of the adaptive immune response[8-9].

Several subsets of DCs in lymphoid and non-lymphoid organs express specialized sets of TLRs[10] and constitutively take up and process antigens[4]. Upon activation, DCs upregulate major histocompatibility complexes (MHC) and co-stimulatory molecules, efficiently present pathogen-derived antigens, and prime antigen-specific naïve T cells. Depending on the pathogen's nature, DCs secrete particular cytokines and shape the adaptive response by inducing defined effector and memory T cell subsets[11]. Classical DCs (cDC) show typical dendritic extensions, take up and present antigens within lymphoid organs[4]. Plasmacytoid DCs (pDC) in contrast have a spherical shape, are found in steady-state BM, spleen and liver, and are recruited to inflamed tissues and draining lymph nodes (LN)[12]. During viral infections, pDCs produce large amounts of type-I interferons, develop a cDC-like morphology[13], and to some extend can present viral antigens[14]. Non-lymphoid tissues, such as the skin, contain DCs[15], which continuously migrate to the LNs, and are referred to as ‘migratory DCs’ (MigDC)[16-17].

Most DCs are replenished from Flt3-receptor expressing progenitors, and lymphoid as well as myeloid $Flt3^+$ progenitors can give rise to DCs[2-3, 18-22]. Along the myeloid branch of hematopoiesis, monocytes, macrophages and DCs arise from the macrophage-dendritic progenitor (MDP)[23-24]. DC-restricted common dendritic progenitors (CDP) are downstream of MDPs[25] and have lost monocyte and macrophage-potential[26-27]. CDPs give rise to pDCs, all subsets of cDCs in lymphoid organs, as well as some DCs within non-lymphoid tissues in organs such as the gut, liver, and kidney[28-30]. cDC and tissue-DC differentiation from CDPs occurs via pre-DCs[31], which have lost pDC potential and currently are considered to be the intermediate DC progenitor that leaves the BM and seeds peripheral lymphoid organs and non-lymphoid tissues[25, 29-30].

Cytokines are involved in regulating DC differentiation from progenitors that express the respective cytokine receptors. Flt3L is an essential cytokine in steady-state DC development[32-35], whereas GM-CSF leads to the generation of cDCs and monocyte-derived inflammatory DCs[15, 36-38]. Although GM-CSF acts predominantly during inflammation, the combined action of Flt3L and GM-CSF is essential for some

non-lymphoid tissue DC-subsets in steady-state dermis[39] and intestine[28]. M-CSF is one further cytokine considered to be involved in DC generation[26, 30, 40-42]. It has long been observed that during severe infections, hematopoietic development is skewed toward lineages of urgent need, a process thought to be indirectly regulated by inflammatory cytokines produced by stromal and immune cells, which sense TLR agonists[8, 43-44]. However, recent findings suggest that human[45-48] and mouse HSPC[49-51] express TLRs and can directly sense PAMPs, which during spreading infections can rapidly reach the highly vasculated BM. Results from available studies suggest that TLR-ligation on HSPCs induces their differentiation to myeloid cells and DCs[46-49, 51], and influences their migratory behavior once circulating through blood, lymph and peripheral tissues[50]. However, no information about TLR expression on DC-restricted progenitors is currently available. We set out to investigate the expression of TLRs on CDPs and hypothesized that TLR activation could influence their migratory behavior.

RESULTS

CDP express *Tlr2*, *Tlr4*, and *Tlr9*

CDPs represent a central intermediate in lymphoid organ and non-lymphoid-tissue DC generation. When screening for expression of *Tlr* mRNA by quantitative real-time polymerase chain reaction (RT-PCR), we compared CDPs and other progenitors from the BM with steady-state DCs from the spleen – the organ that filters the blood for the presence of pathogens. CDPs expressed relative high levels of *Tlr2* mRNA within the range of spleen DCs, and at least 4-fold higher than other BM progenitors (LSK cells, containing hematopoietic stem and multipotent progenitor cells (HSPC), common myeloid progenitors (CMP); granulocyte-macrophage progenitors (GMP); and common lymphoid progenitors, (CLP); see Fig. 1 a). Similarly, CDPs expressed relative high *Tlr9*, at levels lower than spleen DCs, but approximately 10-fold higher than LSK, CMP and GMP (Fig. 1 c). In line with other reports[51], CLPs also expressed relative high levels of *Tlr9*, although somewhat lower than CDPs. LSK cells and GMPs expressed *Tlr4* within the range or higher than spleen DCs, whereas CDPs, CMPs, and CLPs expressed lower, moderate levels (Fig. 1 b). As was shown previously, megakaryocyte-erythrocyte progenitors (MEP) expressed low levels of all TLRs, with values just above the limit of detection. An initial screen showed that

CDPs further expressed *Tlr7* (data not shown); however, levels were lower than on DCs, CLPs or LSK cells. In our hands, none of the available anti-TLR antibodies resulted in convincing stains compared to isotype-matched controls. However, functional TLR2, TLR4, and TLR9 expression could be shown later within this study. We conclude that CDPs in steady-state BM express relative high levels of *Tlr2* and *Tlr9* mRNA, higher than other progenitors tested, and intermediate levels of *Tlr4*.

Chemokine receptor and adhesion molecule expression on CDP

Besides inducing the differentiation of multipotent progenitors towards the myeloid or DC lineage, one study reported that TLR activation of circulating HSPCs stopped further migration, induced local differentiation, and suggested a role of TLRs on influencing the localization of progenitors. For human and mouse HSPCs the chemokine receptor CXCR4 is a key regulator of migration, involved in their BM retention and re-homing upon circulation[52-54], and was further shown to be important for the development of pDCs in BM niches[55]. On the other hand, CCR7 is essential for guiding DCs, as well as naïve, memory and regulatory T cells to the LNs in steady-state and inflammation[16-17]. Therefore, we examined the expression of CXCR4, CCR7, and other migratory molecules on CDP. In steady-state, CDP expressed high levels of *Cxcr4* mRNA and protein compared to naïve T cells, MigDCs, pDCs and cDCs from subcutaneous skin-draining LNs (Fig. 2 a, b). For T cells and steady-state LN DC, surface protein expression of CXCR4 did not always correlate with levels of mRNA expression, probably due to localization in intracellular compartments[56-57] or post-transcriptional regulation.

For *Ccr7*, surface protein clearly correlated with mRNA levels. CDPs in steady-state expressed very low or no Ccr7 mRNA and protein, MigDCs expressed the highest levels, naïve T cells and cDCs intermediate, and pDCs low levels (Fig. 2 c, d). We examined the surface expression of additional migratory markers and found that CDPs were negative for the chemokines receptor CXCR3, mostly negative or low for the integrin CD11c, high for the selectin CD62L, and expressed the selectin-binding glycoproteins CD34 and CD44 (see Supplementary Fig. 1 online). We conclude that in steady-state CDPs express a profile of migratory molecules typical for BM resident progenitors, with relatively high levels of CXCR4, CD34, CD44, and CD62L, but low or no expression of CCR7, CXCR3, or CD11c.

CXCR4 down- and CCR7 upregulation upon direct TLR stimulation

We set out to explore the biological relevance of TLR expression on DC-committed progenitors by stimulating FACS-purified CDPs with TLR-agonists for 12 and 21 h *in vitro*. The results should mirror direct effects of TLR-simulation of CDPs, since no other cells would be present and CDPs would not have sufficient time to differentiate to DCs. All cultures were supplemented with Flt3L, SCF, and M-CSF to ensure CDP survival without introducing any bias towards particular DC subsets. For stimulation, we added PAMPs as established TLR-agonists: The lipooligopeptide Pam_3csk4 for stimulation of TLR2, lipopolysacchride (LPS) for TLR4, and unmethylated CpG-containing oligodeoxynucleotides (CpG) for TLR9. Upon TLR stimulation of CDPs, we observed strong effects in chemokine receptor expression, whereas other genes relevant for DC development, such as transcription factors and cytokine receptors, showed only minor changes in an initial screen (data not shown). We detected significant down-regulation of *Cxcr4* mRNA and up-regulation of *Ccr7* after stimulation for only 12 h with all of the PAMPs tested. Compared to control cultures, we observed the strongest effects with Pam_3csk4 of 11.6-fold upregulation of *Ccr7* and 102-fold down-regulation of *Cxcr4* (Fig. 3 a, c). The next strongest response was detected upon stimulation with CpG, followed by moderate effects with LPS. In line with mRNA expression, we detected differential expression of surface chemokine receptors by flow cytometic analysis after 21 h of TLR stimulation. In control cultures, CDPs expressed CXCR4, whereas upon stimulation with PAMPs, the levels were reduced (Fig. 3 b). For CCR7, CDPs in control cultures were negative, but upregulated CCR7 surface expression in the presence of PAMPs, particularly after stimulation with Pam_3csk4 (Fig. 3 d). In addition, we observed rapid upregulation of CD11c *in vivo* and *in vitro* on CDPs upon stimulation (data not shown). We conclude that CDPs express functional TLRs, whose direct stimulation leads to differential expression of migratory markers, in particular rapid down-regulation of CXCR4 and upregulation of CCR7. This switch from a BM resident to a LN homing phenotype may mobilize CDPs from the BM and lead to the recruitment to draining LN upon TLR stimulation.

CXCR4-dependent mobilization of CDPs from the bone marrow

To test, whether the BM localization of CDPs depends on CXCR4, we injected the CXCR4-antagonist AMD3100, to transiently block CXCR4-interaction with its cognate ligand CXCL12[58-59], and CDP numbers in the BM and peripheral tissues. Despite the BM, no substantial numbers of cells with CDP-phenotype (Lin^- c-kit^{int} $Flt3^+$ M-$CSFR^+$ IL7Rα^- and $CD11c^-$ $MHCcl2^-$) were identified in steady-state peripheral tissues (PBS controls, Fig. 4). Only 1 h after injection with AMD3100, CDP numbers transiently decreased in the BM, increased in the bloodstream, and at 4 h could be detected in spleen and LNs. We conclude that CDP localization in the BM depends on binding of CXCR4 to CXCL12, and once mobilized, CDPs have the machinery to migrate to LNs. To assess whether a similar mobilization of CDPs from the BM would occur during inflammation, probably due to CXCR4-downreculation upon TLR-activation, we intravenously injected PAMPs or PBS as control. 12 h after injection, Lin^- cells expressing Flt3 or M-CSFR, including CDPs, were no longer detectable in BM, blood, and spleen (see Supplementary Fig. 2 online, and data not shown). Similar results were obtained after subcutaneous local administration of PAMPs (data not shown). During ongoing inflammation, increased production and binding of Flt3L and M-CSF could lead to induced receptor internalization or block antibody binding, thus preventing receptor detection. Consequently, since CDPs were characterized by cytokine receptor expression[26], their endogenous numbers and migration during inflammation cannot reliably be determined by surface phenotype.

Enhanced numbers of CDP-derived DCs in inflamed lymph nodes

To further explore the migratory behavior of CDPs and their offspring during inflammation, we adoptively transferred CDPs in non-conditioned hosts and induced inflammation by local injection of CpG into the foot pad. During steady-state (PBS controls), CDPs gave rise to pDCs and cDCs in BM, spleen, and skin-draining LNs 4 days after transplantation (Fig. 5 a), but very few CDP-derived cells with a migratory, skin-derived $CD11c^{int}$ $MHCcl2^{hi}$ MigDC phenotype, were detected in the LNs. No differences were observed comparing PBS-injected and non-injected sides. In contrast, in CpG-treated animals, LNs draining the CpG injected foot pad contained 8.8-fold higher numbers of CDP-grafted cells than LNs on the non-injected side (Fig. 5 c). These were composed of pDCs, and cDCs, which increase 4.9-fold and 6.2-fold, respectively, and of a substantial number of cells with a MigDCs phenotype,

which increased approximately 35-fold, compared to control LNs. This suggests that at least upon CpG-induced local inflammation, CDP-derived cells can enter the skin, give rise to skin DCs, and subsequently migrate to the draining, subcutaneous LNs. It is further noteworthy that the increase of CDP-derived DC subsets (8.8-fold) was higher than the total increase of LN cells, which was 2.9-fold. Compared to steady-state mice (PBS), no clear skewing of pDCs versus cDCs was observed in neither spleen, BM or LNs. To further dissect the increase of CDP-offspring in inflamed settings, we CFSE-labeled CDPs before transplantation to visualize cell divisions. There was no detectable difference in proliferation of CDP-derived cells present in LNs from the injected and the non-injected sides in neither PBS (Fig. 5 b) nor CpG-treated animals (Fig. 5 d); excluding the possibility that higher numbers of DCs were due to increased local proliferation. However, comparing CFSE profiles in LNs (Fig. 5 e), BM (Fig. 5 f), and spleen (data not shown), of PBS and CpG-treated animals illustrated that CDP-offspring had undergone approximately 2 to 3 divisions more in inflamed mice than in steady-state controls. The gating of CDP-derived DC subsets was done similarly as of host-type DCs for each sample; gating and relative frequencies of these are shown in Supplementary Figure 3 online. In summary, our data suggest a direct preferential recruitment of CDPs to inflamed LNs and local pDC and cDC-generation, or a preferential homing of CDP-derived DC. Both of these effects may be due to upregulation of CCR7 upon TLR-triggering by CpG.

DISCUSSION

We show here that common DC progenitors (CDP)[26-27] express relatively high levels of *Tlr2* and *Tlr9* mRNA, and intermediate levels of *Tlr4*, compared to other BM progenitors. Direct activation of CDPs by TLR agonists resulted in rapid upregulation of CCR7 and down-regulation of CXCR4 *in vitro*, suggesting that TLR signaling in DC-restricted progenitors could regulate their migratory behavior. Indeed, the localization of CDPs in the BM was strongly CXCR4-dependent and we observed an 8.8-fold total increase of CDP-derived pDCs, cDCs, and in particular MigDCs in inflamed subcutaneous LNs on day 4 after local injection of CpG.

Published reports previously detected that LSK cells, GMP, and CLP express *Tlr2* and *Tlr4*[49], and that CLP express particular high levels of *Tlr9*[51]. We could confirm TLR expression from earlier studies, and determined even higher levels of

Tlr2 and *Tlr9* expression by CDPs compared to upstream progenitors and steady-state spleen DCs. *Tlr4,* in contrast, was expressed at lower levels by CDP compared to other BM progenitors. The expressed set of TLRs enables CDPs to directly respond to lipoproteins from gram-positive bacteria and enveloped viruses, LPS from gram-negative bacteria, unmethylated CpG-containing DNA from bacteria and DNA viruses, mannans from fungi, as well as the stress response of the attacked host[60], once these products from systemically spreading infections reach the BM.

Previous studies indicated that activation of TLR2 and TLR4 on HSC and upstream myeloid progenitors induces myeloid differentiation towards monocytes and macrophages[46-49]. On the other hand, HSCs stimulated with TLR7/8 agonists[47], and CLPs stimulated with TLR2, TLR4 or TLR9 agonists, as well as during Herpes-Simplex Virus-1 infection *in vivo*[49, 51], differentiated into cDCs and in some cases into pDCs, at the expense of B cell development. The finding that TLR signals can induce myeloid or DC differentiation from HSPC are in line with reports on lineage instruction by cytokine signals[61], which were recently discussed for DC development (M.A. Schmid *et al*., Immunol Rev 234, March 2010, *in press*). We could not detect clear differences in transcription factor expression relevant for the development of specific DC subsets upon TLR activation in CDPs. Instead, we found substantial changes in chemokine receptor expression, hinting for a role of TLRs in regulating DC progenitor migration. One study has suggested that TLR4 on circulating HSPCs activated with LPS stop migrating and locally differentiate into myeloid cells and DCs[50]. In steady-state, CDPs expressed relatively high levels of *Cxcr4* mRNA and protein, but were very low for *Ccr7* mRNA and did not express detectable protein on the surface. Upon *in vitro*-stimulation of CDPs with Pam_3csk4, LPS or CpG, we detected significant down-regulation of *Cxcr4* and upregulation of *Ccr7* mRNA, as well as surface protein expression. Lower levels of CXCR4 surface protein were likely due to down-regulation rather than ligand induced receptor internalization [56-57] since neither progenitors nor maturing DCs are expected to produce CXCL12[62-63] and CXCL12 should accordingly not have been present within the culture system. Since no other cells were present within these short-term cultures, the results show that CDP express functional TLRs and directly respond to TLR agonists by differential expression of chemokine receptors.

The observation that HSPCs, during, inflammation are mobilized from the BM to seed the periphery was recently proposed to be due to down-regulation of

CXCL12 and CXCR4, or their protease cleavage[64]. This was further suggested to indirectly be mediated by inflammatory cytokines such as G-CSF. Considering our *in vitro* findings, we now suggest a direct mechanism where TLR stimulation of CDPs leads to down-regulation of CXCR4, which in turn may lead to DC progenitor mobilization from the BM. For steady-state, we support the view that CDPs predominantly reside within the BM[25-26], as we did not detected convincing numbers of cells with a CD11c$^-$ CDP-phenotype (Lin$^-$ c-kit^{int} Flt3$^+$ M-CSFR$^+$ IL7Rα^- CD11c$^-$ MHCcl2$^-$) in blood, spleen or LNs. Upon injection of CXCR4-antagonist AMD3100, we found that, similar to human and mouse upstream HSPC[58-59], the BM localization of CDP indeed depended on CXCR4. Although endogenous CDP numbers during inflammation can currently not be assessed due to the loss of Flt3 and M-CSFR detection, the rapid down-regulation of CXCR4, and the strong dependency of their BM localization on CXCR4 suggest that CDPs upon TLR triggering may indeed be mobilized from the BM.

Once mobilized to the blood, how do CDP give rise to sufficient numbers of DC in inflamed LNs? DC migration to the LN can occurs via two routes: entry of DCs from blood circulation via high endothelial venuels (HEV)[65] and entry of tissue-resident DC via afferent lymphatics[66], both being dependent on CCR7[67-69]. The ligands for CCR7, CCL21, and CCL19 in steady-state are expressed on HEVs, by stromal cells in the T cell zones, and by lymphatic endothelial cells[69]. LN entry via HEV is the main route of T cells and depends on CD62L and the activation of chemokine receptors as CCR7, which leads to integrin-mediated firm adhesion, and transmigration[70]. The evidence that DCs, for example CD8α^+ cDCs, enter the LN via HEV is indirect and has been only convincingly shown for pDCs[16]. This suggests that in steady-state DC progenitors may enter LNs, and a recent study showed a CD62L-dependent entry of pre-DCs via HEVs and local production of cDCs[25]. Since CDPs in steady-state reside mostly in the BM, pre-DC should indeed be the main circulating cDC-restricted progenitor leaving the BM and seeding peripheral lymphoid organs. However, 4 hours after CDP mobilization via AMD3100, we detected cells with a CD11c$^-$ CDP-phenotype appearing in the LN, indicating that CDPs, once mobilized, carry the potential to enter LNs. In the absence of TLR triggers and 4 hours after mobilization, CDPs had not upregulated CCR7 (data not shown) and their entry into LN could be mediated by CD62L. For B cells, CXCR4 was shown to mediate LN entry in some conditions[71]. Similarly, CXCR4 might have mediated the entry of

mobilized CDPs into LNs, once AMD3100 action ceased. In an inflammatory setting, upon local injection of CpG, we show that CDPs give rise to enhanced numbers of both cDCs and pDCs in inflamed LNs. This raises the possibility that during inflammation CDPs may directly be recruited to reactive LNs via HEVs and locally give rise to cDCs as well as pDCs. During inflammation, HEVs were shown to upregulate CCL21[72], and activated DC secreted CCL19[62-63]. Both effects could contribute to an increase of CCR7-ligand and synergize with the upregulation of CCR7 on CDPs upon TLR activation to mediate their recruitment to reactive LNs. Future research will determine the biological relevance of local DC generation from CDPs compared to direct recruitment of DCs from the blood and if additional migratory markers may play a role in these processes.

The second route of DC entry into LNs is via afferent lymphatics from non-lymphoid tissues. pre-DC were suggested to be the BM-derived progenitor giving rise to some dermal DCs and equivalent DC populations in other non-lymphoid tissues[30]. The final demonstration is still missing, since the detection of skin DC progenitors and their offspring upon adoptive transfer generally seems to be difficult[30] and in our hands, only few events of CDP-derived cells could be detected in the skin of foot pads, 4 h and 4 days after transplant (data not shown). Nevertheless, we demonstrate here that CDPs give rise to substantial numbers of DCs with a migratory phenotype in skin-draining LNs on day 4 after adoptive transfer and local injection of CpG. In steady-state only few CDP-derived MigDCs could be detected. This suggests that during inflammation CDPs, probably via pre-DC, give rise to DCs, which subsequently migrate to the draining LNs. In steady state, the migration of DCs from the skin to the LN via afferent lymphatics was demonstrated to be rather inefficient[73], whereas upon inflammation the number of DCs entering the LN dramatically increased in a CCR7-dependent manner, which was due to upregulation of CCL21 (SLC) on lymphatic endothelial cells[74]. CDPs may consequently also give rise to skin DCs in steady-state, but their subsequent migration to LNs may be relatively low. DC migration to the LNs is amplified by the upregulation of CCR7 on activated DCs[75-78]. An increased migration of CDP-derived cells and enhanced numbers of MigDCs in inflamed LNs could also be mediated by an upregulation of CCR7 upon TLR stimulation. However, the initial entry of CDPs into the skin in steady state or inflammation may rather be due to signaling via other chemokine, such as CCR2, which was shown to be essential for the entry of monocyte into

peripheral tissues[79]. Since no clear differences were detectable comparing the ratio of CDP-derived pDCs and cDCs in BM, spleen, or LNs in steady state and inflammation, our data suggest that TLR triggering may rather enhance DC progenitor migration and entry into different organs, rather than their differentiation towards one or the other DC subset.

Previous studies suggested an induced proliferation of upstream progenitors upon TLR triggering[48-50]. In our experiments, we did not detect an increase in local proliferation of CDPs or their offspring within the LNs of the CpG-injected versus the control side of the animals. Nevertheless, when comparing CpG-injected versus PBS-treated control animals, the inflamed animals showed higher proliferation of CDPs in all organs. DC progenitor proliferation might thus be regulated by systemic effects, for example by secreted cytokines such as Flt3L, rather than TLR triggering. To what extent increased survival may contribute to increased DC numbers in inflamed LNs and whether the combination of different PAMPs may lead to synergistic effects during an ongoing infection remains to be determined.

In conclusion, we show that CDPs can directly sense the presence of PAMPs in the BM via TLRs, change their chemokine receptor expression profile, which may result in their mobilization into the blood circulation and migration to inflamed LNs. Extending previous studies suggesting migratory arrest of circulating HSC upon TLR encounter[50], we suggest a novel TLR-dependent mechanism that induces active DC-restricted progenitor migration from the BM to reactive LNs. These effects may help to maintain sufficient DC numbers in reactive LNs during the ongoing immune response and to restore DC homeostasis, once the inflammation ceases.

METHODS

Mice. Sex- and age-matched, 6- to 8-week-old C57BL/6-KA-CD45.1 or C57BL/KA-CD45.2 mice were used, if not stated otherwise. Of these, F1 mice CD45.1xCD45.2 were generated by interbreeding. All mice were bred and maintained at the Institute for Research in Biomedicine or the University Hospital Zurich animal facility. Mice were treated in accordance with guidelines of the Swiss Federal Veterinary Office and experiments were approved by the '*Dipartimento della sanità e della socialità del cantone Ticino*' or the 'Gesundheitsdirektion Kanton Zuerich, Veterinaeramt'.

Antibodies. All antibodies were purchased from eBiosciences, unless otherwise stated. The following monoclonal antibodies conjugated to various fluorochromes or biotin were used: CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70), CD11c (N418), CD16/CD32 FcgR-III/II (2.4G2, Becton Dickinson), CD19 (ID3), CD34 (Ram34), CD45RA (14.8; Becton Dickinson), CD45.1 (A20), and CD45.2 (104), CD44 (IM7, Becton Dickinson), CD62L (MEL-14), CCR7 (4B12), CXCR3 (220803; R&D), CXCR4 (2B11), B220 (RA3-6B2), c-kit (ACK2), Flt3 (A2F10), Gr-1 (RB6-8C5), IL7Rα (A7R34), M-CSFR (AFS98), MHCcl2 (M5/114.15.2; Becton Dickinson), NK1.1 (PK136; Becton Dickinson), Sca-1 (D7; or E13-161.7, Becton Dickinson), Ter119 (Ter119). Biotinylated antibodies were visualized with fluorochrome-conjugated to streptavidin. Cells were stained at 4°C in FACS-buffer (PBS, 2% fetal bovine serum (FBS), 2 mM EDTA) or for chemokine-receptors at room temperature after blocking Fc-receptor binding with anti-CD16/CD32. For isotype-matched controls, all other markers were stained and populations were gated similarly to samples stained with specific antibodies.

Cell preparation and flow cytometry. Single cell suspensions of BM mononuclear cells were obtained by smashing long bones and spine with mortar and pestle, filtering through nylon meshes, and performing density centrifugation with Histopaque-1077 (Sigma-Aldrich). Spleens and LNs were mashed and digested for 30 min at 37°C with 10 U/ml DNase-I and 1 mg/ml CollagenaseD (Roche). Red blood cells were lysed from spleen and blood samples in 150 mM ammonium-chloride and 10 mM potassium-hydrogen-carbonate. Dead cells were excluded by propidium iodide staining (Invitrogen). For flow-cytometric analysis, cells were acquired on a FACSCanto (Becton Dickinson) and analyzed with the use of FlowJo software (TreeStar). Before cell sorting, samples were immuno-magnetically pre-selected. For BM progenitor populations, cells positive for lineage antigens (Lin; CD3ε, CD4, CD8α, CD19, CD11b, B220, Gr-1, NK1.1, Ter119) were stained with PE-Cy5-conjugated antibodies, magnetically labeled with anti-Cy5-MicroBeads (Miltenyi) and negatively selected with MACS LS-columns and MidiMACS Separators (Miltenyi). For sorting spleen and LN DCs, samples were stained with anti-CD11c-APC, labeled with anti-APC-MicroBeads (Miltenyi) and enriched for CD11c. Cell populations were sorted with a FACSAria1 (Becton Dickinson) according to the following cell surface phenotypes: LSK cells, Lin^- c-kit^{hi} Sca-1^+; CMP^{80}, Lin^- IL7Rα^- c-kit^{hi} Sca-1^- $CD34^+$

$CD16/32^{-}$; GMP, Lin^{-} $IL7R\alpha^{-}$ $c\text{-}kit^{hi}$ $Sca\text{-}1^{-}$ $CD34^{+}$ $CD16/32^{+}$; MEP, Lin^{-} $IL7R\alpha^{-}$ $c\text{-}kit^{hi}$ $Sca\text{-}1^{-}$ $CD34^{+}$ $CD16/32^{-}$; $Flt3^{+}$ $CLP^{81\text{-}82}$, Lin^{-} $c\text{-}kit^{int}$ $Flt3^{+}$ $IL7Ra^{+}$; CDP^{26}, Lin^{-} $c\text{-}kit^{int}$ $Flt3^{+}$ $IL7Ra^{-}$ $M\text{-}CSFR^{hi/low+}$ from the BM; pDC, $CD19^{-}$ $NK1.1^{-}$ $CD11c^{int}$ $CD45RA^{+}$; cDC, $CD19^{-}$ $NK1.1^{-}$ $CD45RA^{-}$ $CD11c^{hi}$ $MHCcl2^{+}$ from spleen; naïve T cells (including central memory T cells), $CD3^{+}$ $CD4^{+}$ $CD62L^{+}$; MigDC, $CD19^{-}$ $CD45RA^{-}$ $CD11c^{int}$ $CD40^{hi}$ $MHCcl2^{hi}$; pDC, $CD19^{-}$ $CD11c^{int}$ $CD45RA^{+}$; cDC ,$CD19^{-}$ $CD45RA^{-}$ $CD11c^{hi}$ $CD40^{int}$ $MHCcl2^{int}$ from subcutaneous LNs.

Quantitative real-time polymerase chain reaction analysis (RT-PCR). Cell populations sorted *ex vivo* or after *in vitro* culture were resuspended in TRIzol-LS reagent (Invitrogen) and RNA was extracted. Remaining DNA was eliminated by DNase-I treatment using the DNA-free kit (Applied Biosystems). For cDNA-synthesis equal amounts of RNA were reverse transcribed using SuperScript-III (Invitrogen). Quantitative RT-PCR was performed on a 7900HT Fast System (Applied Biosystems) using Taqman primers & probes for *Rn18s* (Hs99999901_s1), mouse *Tlr2* (Mm00442346_m1), *Tlr4* (Mm00445274_m1), *Tlr9* (Mm0046193_m1), *Cxcr4* (Mm01292123_m1), and *Ccr7* (Mm00432608_m1). Results were normalized to *Rn18s*.

***In vitro* TLR-agonist stimulation.** FACS-sorted CDPs were cultured 12 h for analysis of mRNA expression by quantitative RT-PCR or 21 h for surface protein expression in RPMI-1640 supplemented (Gibco) with 10% FBS (Gibco), 2% Penicillin-Streptomycin (Invitrogen), GlutaMAX (Invitrogen), 50 ng/ml mouse Flt3L, 10 ng/ml SCF, and 20 ng/ml M-CSF (R&D). Cultures were stimulated with 10 μg/ml Pam_3csk4 (Invivogen), 10 μg/ml ultrapure LPS from E.coli 0111:B4 (Invivogen) or 5 μg/ml CpG-ODN 1826 (Microsynth).

***In vivo* analysis of endogenous progenitors.** For *in vivo* analysis of endogenous progenitors, WT mice were injected with 100 μg of CXCR4-antogonist AMD3100 (Plerixafor, Genzyme) subcutaneously or 35 μg of Pam_3csk4, LPS or CpG-ODN 1826 intravenously. Control mice received equal volumes of PBS. For flow cytometric analysis of progenitors, at least $1.5x10^6$ PI^{-} cells were acquired from BM, approximately $3.8x10^6$ from spleen and LNs, and all events from approximately 750 μl of blood.

***In vivo* adoptive transfer assays.** 2-3x10^5 CDPs were FACS-sorted from BM of CD45.1^+ female mice, labeled for 8 min with 2 μM CFSE at 37°C, and transplanted intravenously into three week old CD45.2^+ or CD45.1xCD45.2^+ F1 female non-conditioned hosts. Inflammation was induced by injecting 25 μg GpG-ODN 1826 subcutaneously into the foot pad as indicated. Steady-state transplanted control animals were injected with PBS. For analysis, BM and spleen samples were immuno-magnetically pre-enriched for donor-cells expressing the CD45.1 allele by staining with CD45.1-PE and anti-PE MicroBeads (Miltenyi). For data acquisition, approximately 3.5x10^6 PI^- events from BM and spleen samples before enrichment, the enriched samples, and all cells obtained from the draining LNs were acquired.

Statistics. The data was analyzed for statistical significance with the use of two-tailed unpaired t tests using Prism 4 software (GraphPad). Graphs show the means plus (or respectively minus) standard error of mean. P was considered significant at values less than 0.05.

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AUTHORS CONTRIBUTIONS

M.A.S designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. D.K. helped performing experiments, collected and discussed data, and edited the manuscript. M.G.M. directed the study, discussed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS STATEMENT

The authors declare no competing financial interests.

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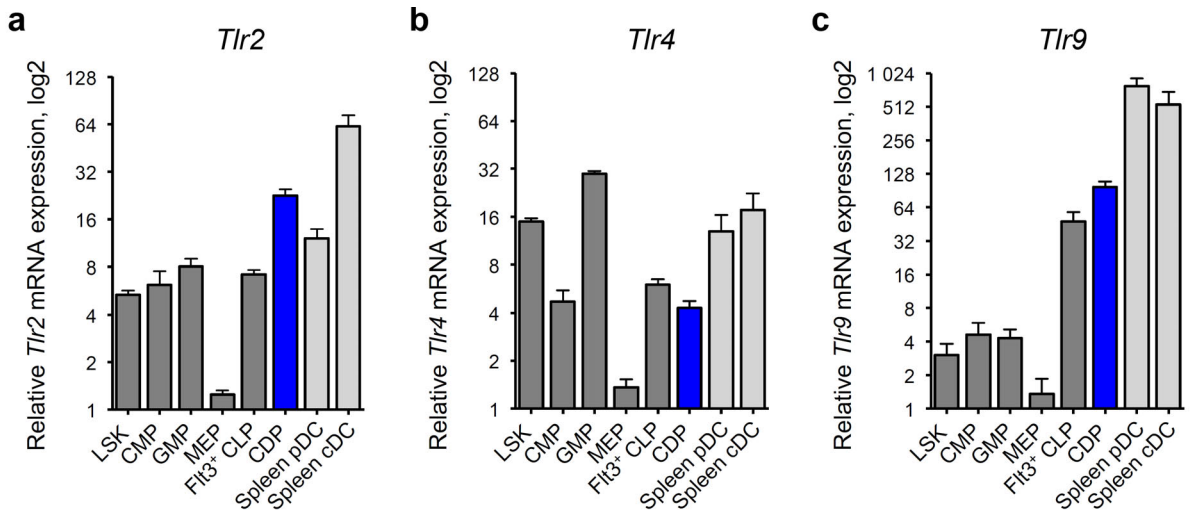


Figure 1. Common dendritic progenitors (CDP) express relatively high levels of *Tlr2*, *Tlr4,* and *Tlr9*. Relative mRNA expression of *Tlr2* (a), *Tlr4* (b) and *Tlr9* (c) is shown for hematopoietic progenitor populations (dark grey) and CDPs (blue) isolated from mouse BM, and DCs isolated from steady-state spleens (light grey). Populations were FACS-sorted and mRNA expression determined by quantitative RT-PCR. Graphs show means and standard error of mean of at least four independent experiments plotted on a bi-logarithmic scale (log2).

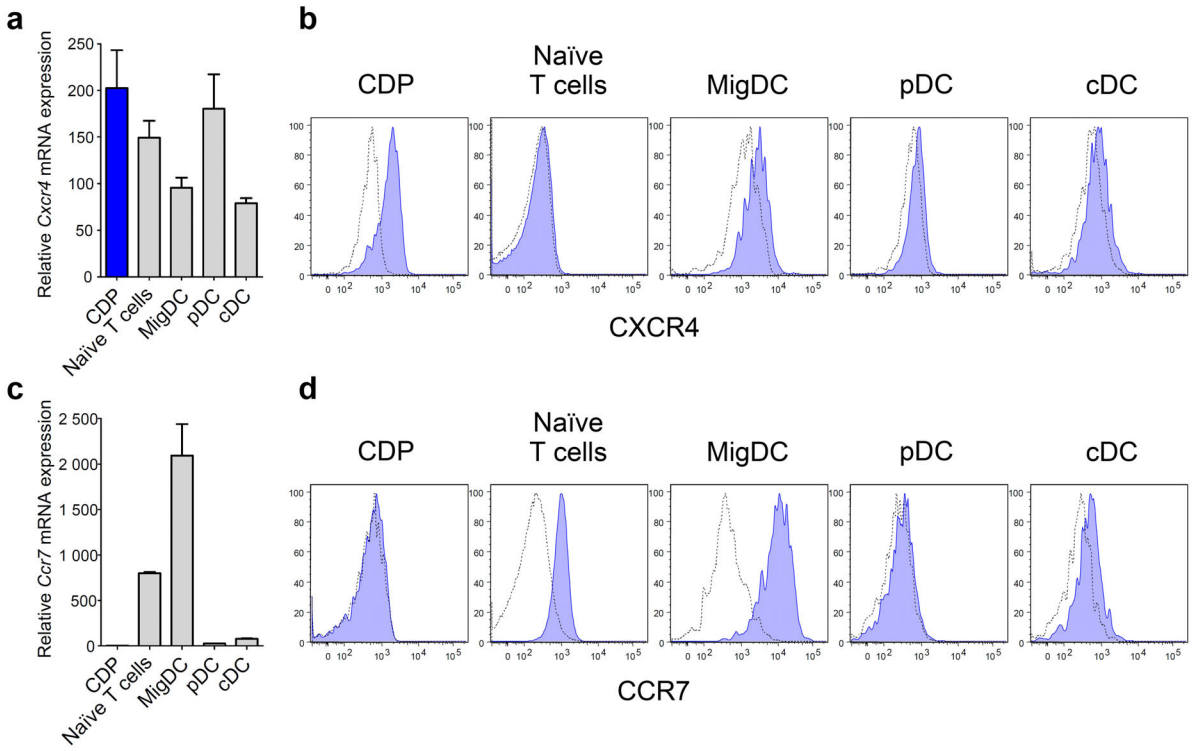


Figure 2: CDP in the steady-state BM express relatively high levels of *Cxcr4*, but no or low *Ccr7*. CDPs isolated from steady-state BM were assessed for expression of chemokines receptors *Cxcr4* (a, b) and *Ccr7* (c, d), compared to defined populations from subcutaneous LNs. Bar graphs show relative mRNA expression for *Cxcr4* (a) and *Ccr7* (c) of CDPs (blue), and naïve T cells, migratory dendritic cells (MigDC), plasmacytoid DC (pDC), and classical DC (cDC) from LNs (grey) isolated by FACS. mRNA expression levels were evaluated by quantitative RT-PCR. Histogram overlays show surface protein expression *ex vivo* of CXCR4 (b) and CCR7 (d) assessed by flow cytometry for the same populations. Specific antibody-stains are shown in filled blue histograms, isotype-matched controls in dotted lines. Bar graphs show means and standard errors of mean of at least three independent experiments (a, c), histograms show one representative of at least three independent experiments (b, d).

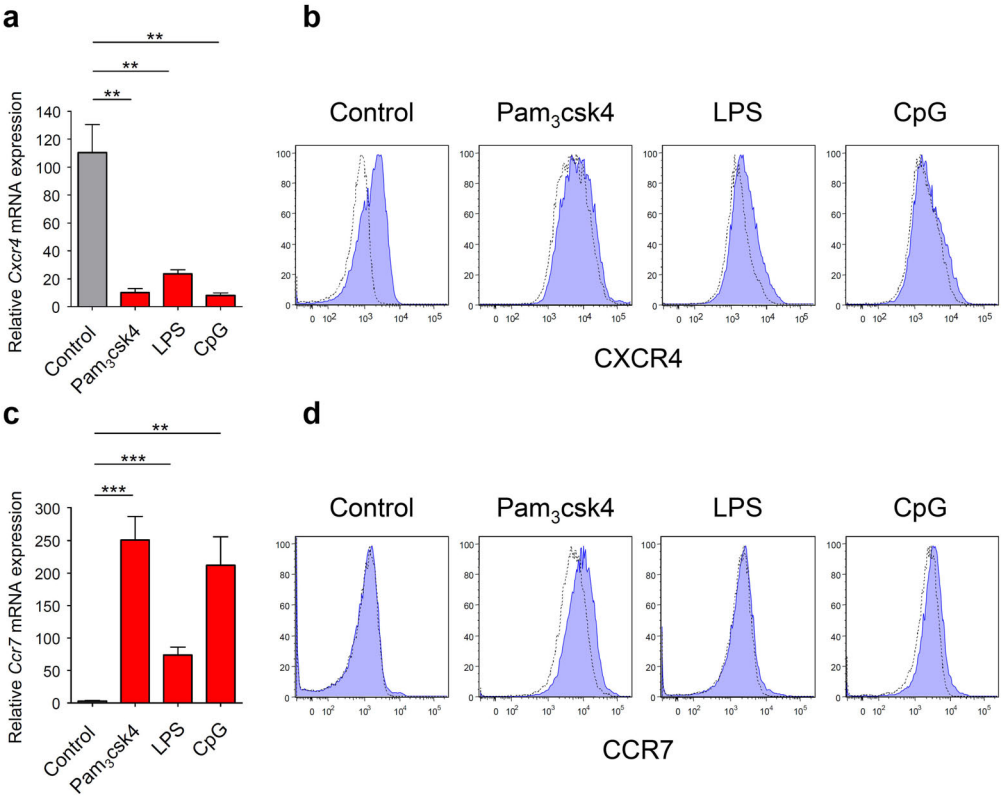


Figure 3. CDPs directly sense TLR-agonists, rapidly down-regulate CXCR4 and upregulate CCR7. FACS-sorted CDPs were cultured in the presence of cytokines alone (control) or stimulated with the PAMPs Pam_3csk4, LPS or CpG. mRNA was isolated and assessed by quantitative RT-PCR 12 h later (a, c). Alternatively protein surface expression was determined by flow cytometry after 21 h of culture (b, d). Bar graphs show relative expression of mRNA for *Cxcr4* (a) and *Ccr7* (c) in cultures with cytokines only (grey) or with TLR-agonists (red). Histograms show protein surface expression (blue filled) for CXCR4 (b) and CCR7 (d), overlaid with isotype-matched controls (dotted lines). Bars graphs represent means and standard errors of mean of four (a) or five (c) independent experiments. Histogram overlays show one representative out of two independent experiments (b, d).

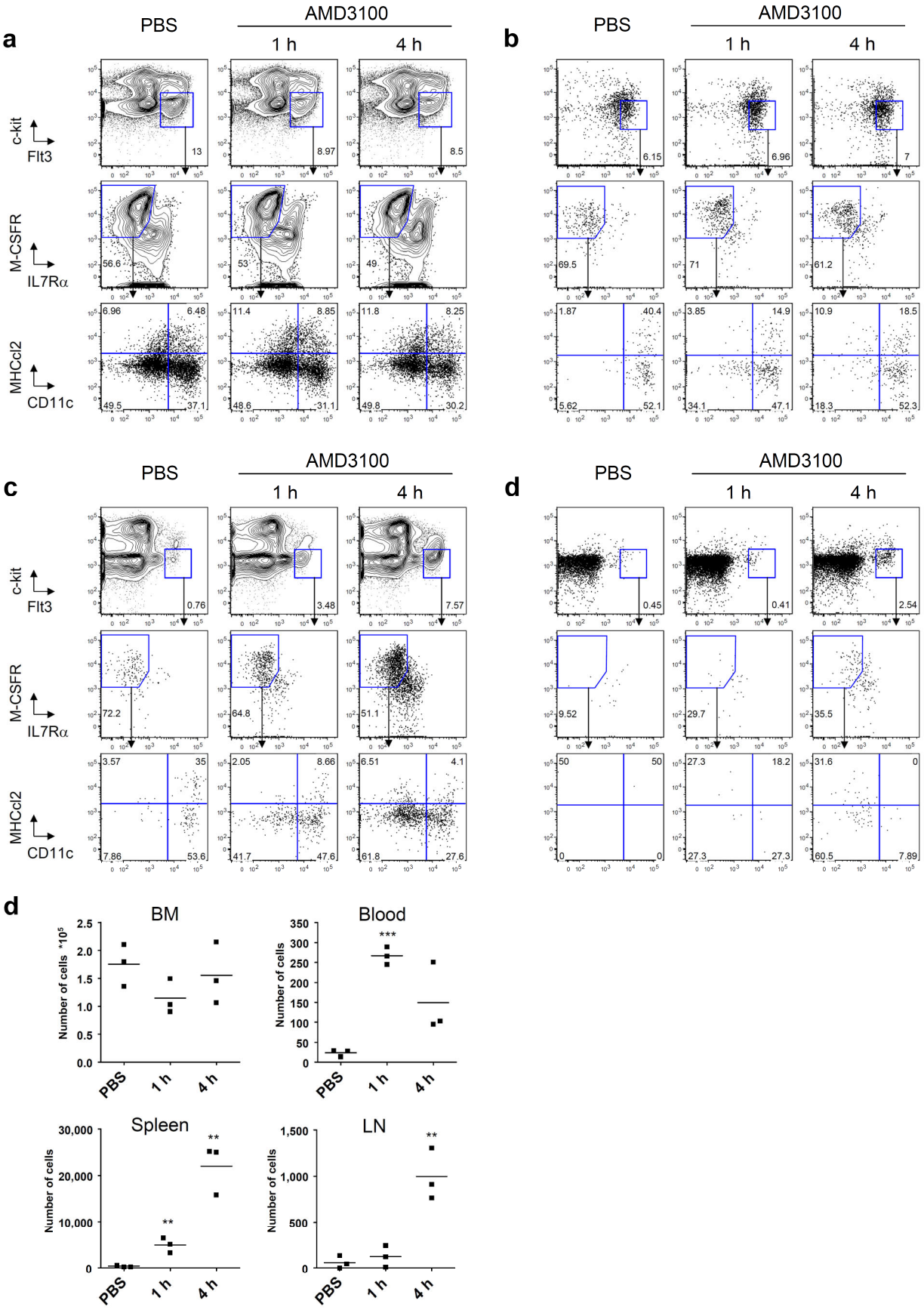

a
PBS
AMD3100
1 h
4 h
c-kit
Flt3
M-CSFR
IL7Rα
MHCcl2
CD11c
13
8.97
8.5
56.6
53
49
6.96
6.48
11.4
8.85
11.8
8.25
49.5
37.1
48.6
31.1
49.8
30.2
b
6.15
6.96
7
69.5
71
61.2
1.87
40.4
3.85
14.9
10.9
18.5
5.62
52.1
34.1
47.1
18.3
52.3
c
0.76
3.48
7.57
72.2
64.8
51.1
3.57
35
2.05
8.66
6.51
4.1
7.86
53.6
41.7
47.6
61.8
27.6
d
0.45
0.41
2.54
9.52
29.7
35.5
50
50
27.3
18.2
31.6
0
0
0
27.3
27.3
60.5
7.89
d
BM
Blood
Spleen
LN
Number of cells *10⁵
Number of cells
PBS
1 h
4 h
***
**

Figure 4.

Figure 4. CXCR4-antagonist AMD3100 mobilizes CDPs from the BM, leading to their circulation in blood and entry into spleen and LNs. The frequency of CDPs in BM (a), blood (b), spleen (c), and subcutaneous LNs (d) was assessed by flow cytometry 1 h after injection of PBS or 1 and 4 h after injection of CXCR4-antagonist AMD3100. The top row of plots shows Lin^- cells and the successive gating (top to bottom) to detect cells with CDP-phenotype as Lin^- c-kit^{int} $Flt3^+$ M-$CSFR^+$ IL7Rα^- $CD11c^-$ and $MHCcl2^-$. Plots from one representative out of three independent experiments are shown. Absolute numbers of CDPs are shown for individual experiments in (d); mean values (line) and level of significant difference to PBS controls (*) are indicated.

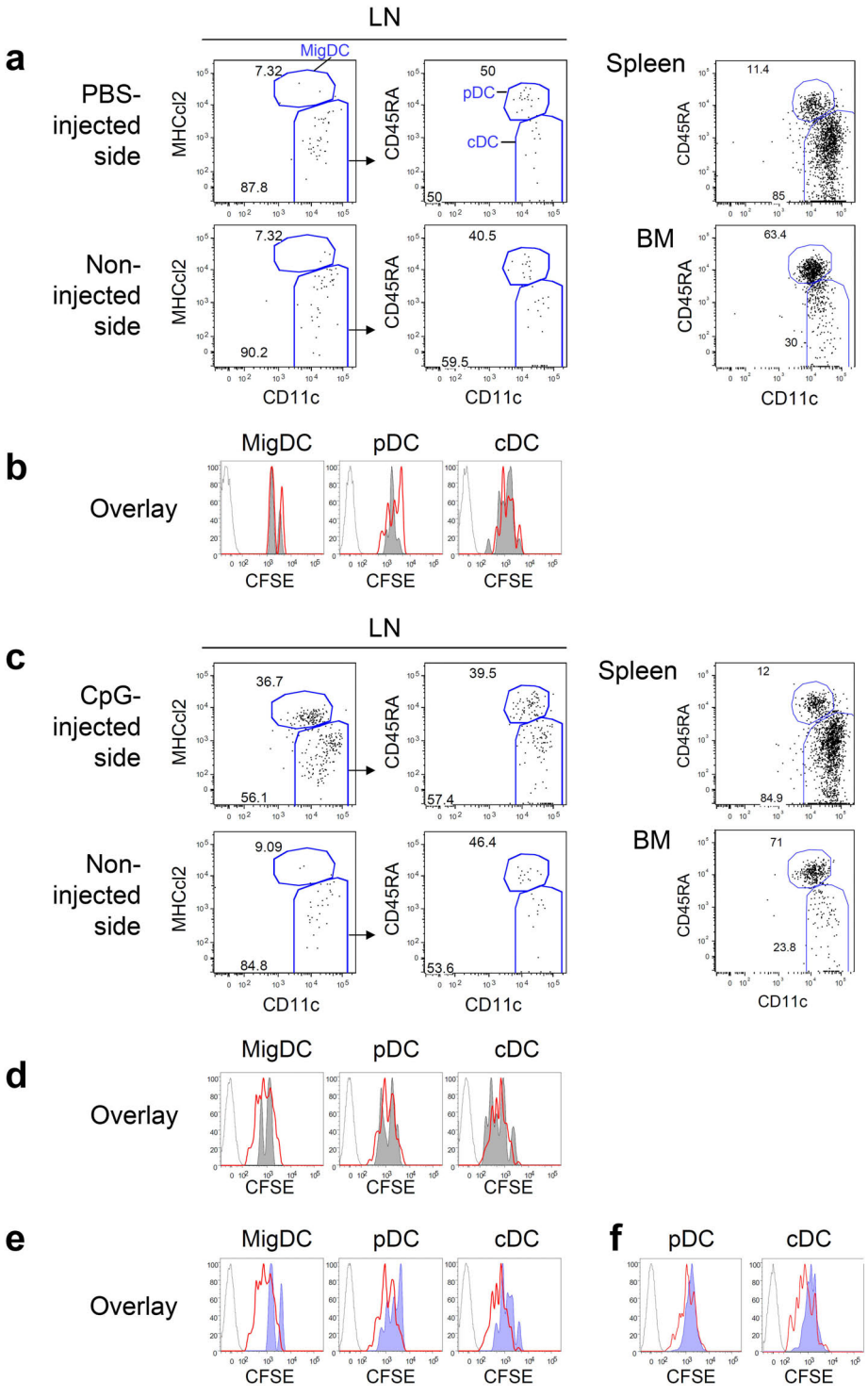
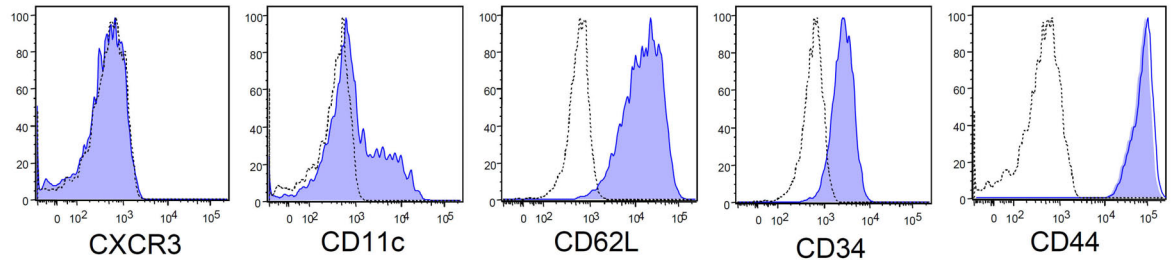

a
LN
MigDC
PBS-injected side
Non-injected side
MHCcl2
CD11c
CD45RA
pDC
cDC
7.32
87.8
50
90.2
40.5
59.5
Spleen
BM
11.4
85
63.4
30
b
Overlay
MigDC
pDC
cDC
CFSE
c
CpG-injected side
36.7
56.1
39.5
57.4
9.09
84.8
46.4
53.6
12
84.9
71
23.8
d
e
f

Figure 5.

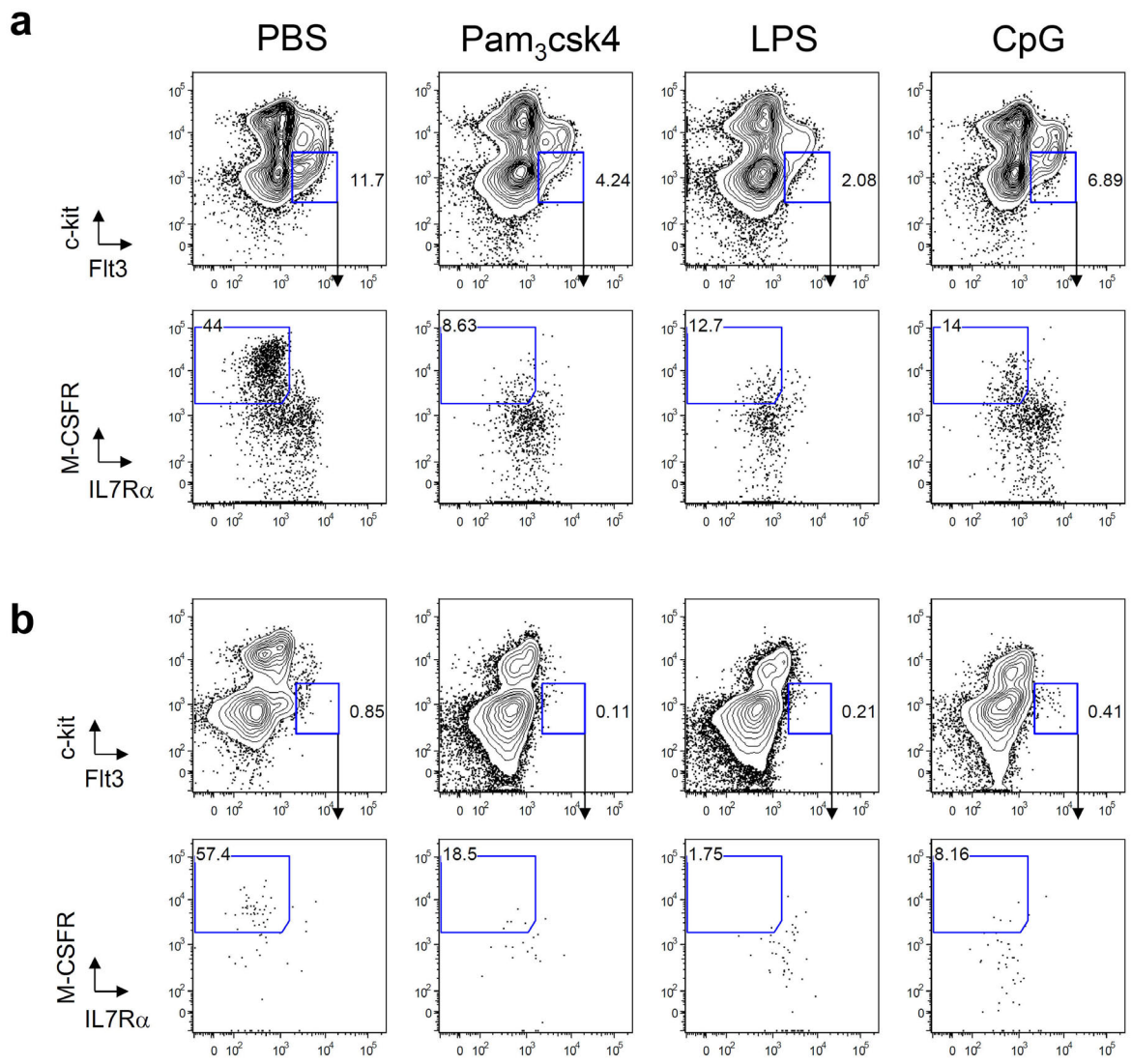
Figure 5. Adoptively transferred CDPs give rise to enhanced numbers of migratory DCs, pDCs and cDCs in inflamed LNs upon local injection of CpG. CFSE-labeled CDPs were adoptively transferred into CD45.1xCD45.2 hosts, which were at the same time injected with PBS (a, b) or with 25 μg CpG (c, d) into the left front foot pad. Dot plots show CDP-derived cells on day 4 gated as $CD19^-$ $NK1.1^-$ $CFSE^+$ $CD45.1^+$ $CD45.2^-$ in LNs, spleen and BM (a, c). For LNs, those draining the front foot pads of the injected and those of the non-injected side are shown. DC-subsets were gated for migratory DCs (MigDC), pDCs, and cDCs, as indicated. Histogram overlays show CFSE-dilution to assess proliferation history comparing draining LNs from injected (red line) and non-injected sides (grey filled) of an animal after PBS (b) or CpG treatment (d). Overlays comparing LNs draining the injected sides of CpG-treated (red line) and PBS-treated animals (blue filled) are pictured in (e), and from the BMs in (f). Data represent one out of three independent experiments.

Schmid *et al*.

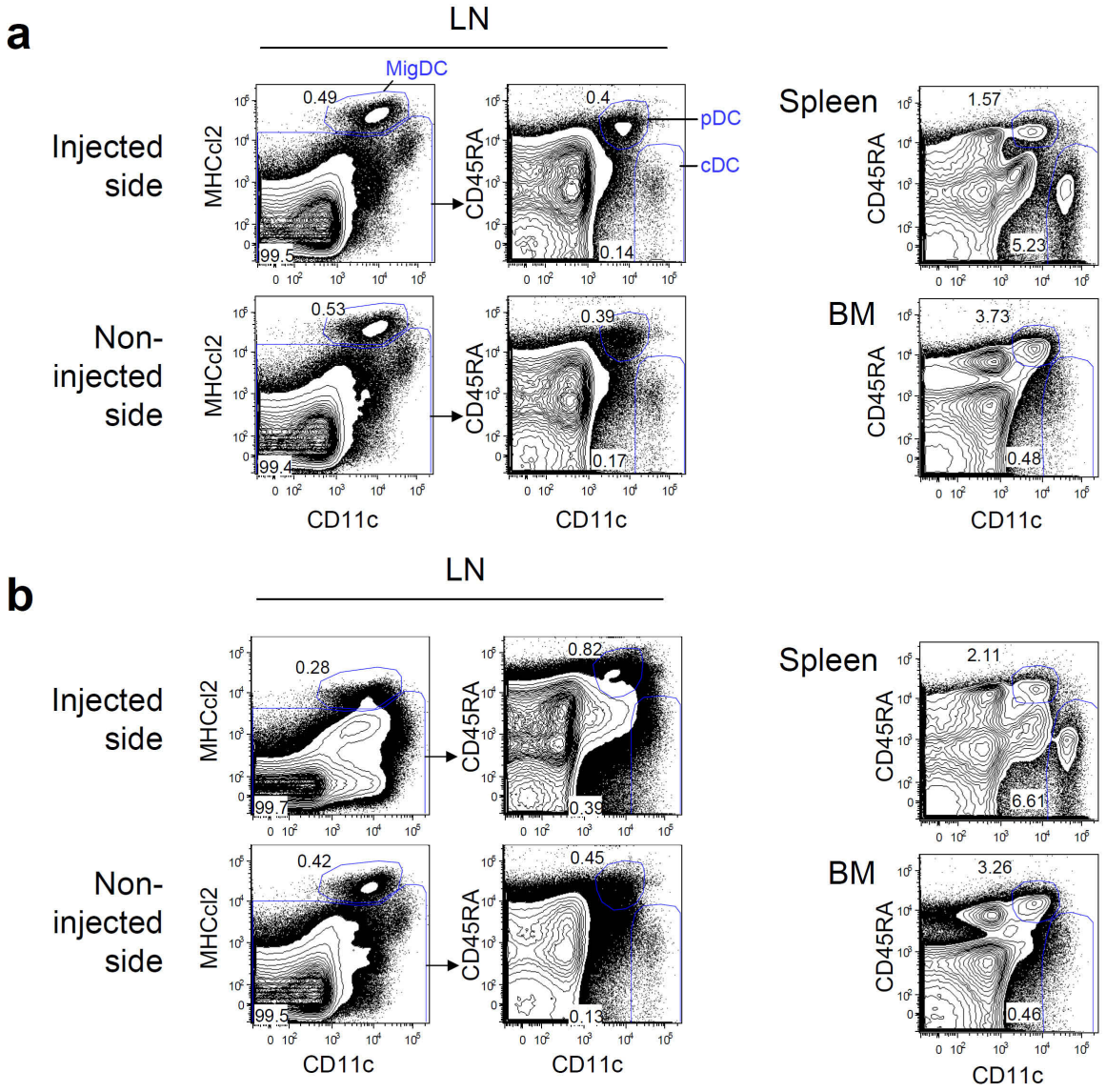
Supplementary material



Supplementary Figure 1. Expression of cell adhesion molecules by CDPs in steady-state. Histogram overlays show antibody surface stains of BM sample for chemokines receptor CXCR3, integrin CD11c, selectin CD62L, and selectin-binding glycoproteins CD34 and CD44 (filled blue) and isotype-matched controls (dotted lines) gated Lin^- c-kit^{int} $Flt3^+$ M-$CSFR^+$ $IL7R\alpha^-$. Representative results of three independent experiments are shown.



Supplementary Figure 2. Cells with CDP phenotype are no longer detectable 12 h after induction of systemic inflammation via intravenous injection of PAMPs. Pam_3csk4, LPS or CpG were injected intravenously or PBS for control animals. The frequency of cells with CDP-phenotype in BM (a), blood (not shown), and spleen (b) was analyzed by flow-cytometry after 12 h by successive gating (top to bottom) as Lin^- c-kit^{int} $Flt3^+$ M-$CSFR^+$ $IL7R\alpha^-$.



Supplementary Figure 3. Analysis of host-derived DCs during CpG-induced inflammation. Contour plots show flow cytometric analysis of host-derived cells gated $CD19^-$ $NK1.1^-$ $CFSE^-$ $CD45.2^+$ from experiments described in Figure 5. Panels show results for axillary and brachial LNs from injected and non-injected sides and for spleen and BM from PBS (a) or CpG-treated animals (b). The indicated gating of host DC-subsets in LNs was used to determine equivalent gating of MigDCs, pDCs, and cDCs derived from CDPs.

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Instructive cytokine signals in dendritic cell lineage commitment

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Summary: Clarifying the signals that lead to dendritic cell (DC) development and identifying cellular intermediates on their way to DC differentiation are essential steps to understand the dynamic regulation of number, localization, and functionality of these cells. In the past decade, much knowledge on cytokines, transcription factors, and successive progenitors involved in steady-state and demand-adapted DC development was gained. From the stage of multipotent progenitors, DCs are generated from $Flt3^+$ intermediates, irrespective of lymphoid or myeloid commitment, making fms-related tyrosine kinase 3 ligand one of the major regulators for DC development. Additional key cytokines involved are granulocyte–macrophage colony-stimulating factor (GM-CSF) and M-CSF, with each being essential for particular DC subsets and leading to specific activation of downstream transcription factors. In this review, we seek to draw an integrative view on how instructive cytokine signals acting on intermediate progenitors might lead to the generation of specific DC subsets in steady-state and during inflammation. We hypothesize that the lineage potential of a progenitor might be determined by the set of cytokine receptors expressed that make it responsive to further receive lineage instructive signals. Commitment to a certain lineage might consequently occur when lineage-relevant cytokine receptors are further upregulated and others for alternative lineages are lost. Along this line, we emphasize the role that diverse microenvironments have in influencing the generation of DC subsets with specific functions throughout the body.

Keywords: *dendritic cells development, common dendritic cells progenitor, fms-like tyrosine kinase 3 ligand, cytokines, lineage instruction, microenvironment*

Introduction

Dendritic cells (DCs) have a short half life in lymphoid organs and continuously need to be replenished from bone marrow hematopoietic stem cells (HSCs) and progenitor cells (1–4). Diverse subsets of DCs in various tissues throughout the body carry out multiple functions in steady-state and during ongoing immune responses. These functional differences are topics of other reviews within this issue; however, we briefly introduce the two major lymphoid tissue-resident DCs populations: classical DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs are the typical DCs displaying dendritic extensions and having antigen-presentation function in steady-state (5, 6). Also known as lymphoid organ-resident DCs, cDCs are derived from blood-borne progenitors and are found in the

thymus, spleen, and lymph nodes, where their actions are restricted to the organ in which they reside. cDCs express high levels of CD11c and intermediate to high levels of major histocompatibility complex (MHC) class II. Unlike cDCs, pDCs lack dendrites but have a plasmacytoid shape that has given them their name (7). Maturation of pDCs mostly occurs in the bone marrow, followed by their circulation in the blood and exit into the tissues (8). pDCs are uniquely equipped to respond to viral infection through their expression of Toll-like receptor 7 (TLR7) and TLR9, which recognize single-stranded RNA and double-stranded, unmethylated CpG-containing oligodeoxynucleotides, respectively (3, 7). Upon ligation of these TLRs, pDCs produce large amounts of type I interferons (IFNs), which act in both an autocrine and paracrine manner to induce an anti-viral state. pDCs express lower levels of MHC class II and are much less efficient at inducing T-cell proliferation compared with cDCs (9, 10). However, during certain inflammatory settings, for instance influenza infection, pDCs are activated and can further differentiate into a distinct CD8α^+ DC subset in the spleen and can more efficiently prime T cells (3, 11, 12).

In this review, we describe significant findings in DCs development that have shaped our current view on respective progenitors and their cytokine requirements. We draw an integrative model where external signals provided in defined microenvironments specifically influence the development of DCs subsets with specialized functions.

Cytokines in DCs development

The differentiation of HSCs to mature hematopoietic cells requires the integration of environmental signals surrounding the cell (13). In their microenvironments, hematopoietic progenitors interact via receptors with ligands present in the extracellular matrix. In these developmental niches, cytokines can act locally as secreted factors or in membrane-bound forms in direct cell-to-cell contacts. In addition, cytokines can act distantly from where they were produced by travelling along the bloodstream or lymph vessels.

To maintain homeostasis in the DC compartment, DCs with rapid turnover continuously need to be replaced from progenitors. Granulocyte–macrophage colony-stimulating factor (GM-CSF) was the first cytokine discovered that efficiently supported DCs differentiation *in vitro* (14–16). Addition of GM-CSF to monocyte or bone marrow cultures leads to robust differentiation of DCs; however, these DCs are morphologically and functionally distinct from *ex vivo*-isolated cDCs (17). GM-CSF-generated DCs are larger and contain more granules than cDCs and also produce higher amounts of tumor necrosis factor (TNF)-α and nitric oxide after stimulation with pathogen-associated molecular pattern recognition receptor ligands. Therefore, they correlate with inflammatory TNF/inducible nitric oxide synthase-producing DCs (Tip DCs) that are derived from monocyte precursors. Similar to GM-CSF, addition of fms-like tyrosine kinase 3 ligand (Flt3L) to *in vitro* bone marrow cultures results in the generation of DCs (18). In contrast to GM-CSF, Flt3L-driven cultures produce both cDCs and pDCs. This Flt3L-driven pDCs and cDCs generation exclusively occurs from bone marrow progenitors but not from monocytes, and more closely resemble the steady-state, *ex vivo*-isolated counterparts (17, 19).

Although the protocol of generating DCs in cultures from bone marrow cells or monocytes supplemented with GM-CSF is commonly used, *in vivo*, GM-CSF-deficient mice or mice lacking the GM-CSF receptor common β chain have only minor decreases in splenic cDCs compared with wildtype mice and a maximal threefold reduction in lymph node cDCs (20). Transgenic mice overexpressing GM-CSF showed only a small increase in cDCs. Thus, the data suggest that GM-CSF primarily acts during inflammation and produces inflammatory DCs but is less relevant for steady-state DC maintenance. There is additional evidence for this hypothesis: GM-CSF-supplemented bone marrow cultures generate monocyte-derived Tip DCs; GM-CSF is undetectable in the blood in steady-state; the absence of GM-CSF seems to have little effect on steady-state cDCs maintenance in the presence of compensatory cytokines; and lastly, adoptively transferred monocytes generate only non-lymphoid tissue DCs and DCs in the spleen during inflammation (17, 21–23).

Unlike mice lacking GM-CSF-mediated signaling, mice with a targeted gene deletion of Flt3L or Flt3 (CD135, Flk2) have severely reduced cDCs, pDCs, and interstitial dermal DCs (24–26). In addition, repetitive injection or conditional expression of Flt3L leads to massive expansion of cDCs, pDCs, and myeloid cells but not B or T lymphocytes (27–29). To investigate the concerted action of GM-CSF and Flt3L, we generated GM-CSF/Flt3L double knockout mice and demonstrated that in the absence of the compensatory action of Flt3L, GM-CSF is essential for the generation of the CD11b$^+$ subset of dermal DCs (26). Thus, at steady-state, although GM-CSF is not measurable in the serum, local amounts of GM-CSF contribute synergistically with Flt3L to the homeostasis of distinct DCs subsets, while Flt3L alone is critical for both lymphoid and non-lymphoid tissue DCs (24, 26).

In addition to Flt3L and GM-CSF, studies within the last 5 years have demonstrated a role for M-CSF in DCs develop-

ment. The first indication that M-CSF and its receptor (M-CSFR) (CD115) contribute to DCs development was the discovery that M-CSFR-deficient mice completely lack Langerhans cells (LCs) (30). Surprisingly, mice lacking M-CSF ($Csf1^{op/op}$ mice), because of a homozygous mutation in the coding region, had normal levels of LCs. However, after ultraviolet-treatment, the repopulation kinetics of LCs were slower in the op/op mice than in wildtype mice. The normal numbers of LCs in steady-state op/op mice indicate that another growth factor may bind and signal through the M-CSFR, and this factor has been recently identified as IL-34 (31). In addition to LCs, use of M-CSFR-green fluorescence protein (GFP) reporter mice (32) demonstrated expression of the GFP transgene in both cDCs and pDCs, although the surface staining of M-CSFR was very low compared with the reporter gene expression (32, 33). In accordance with these results, op/op mice have on average two- and threefold reductions in splenic cDCs and pDCs, respectively (33). Additionally, both pDCs and cDCs were found in M-CSF-cultured total bone marrow and progenitor cultures in the absence of Flt3L (34). Furthermore, *in vivo*, pDCs and cDCs were increased in Flt3L-deficient mice treated with M-CSF, demonstrating that M-CSF alone under certain conditions can drive DCs generation independent of Flt3L.

DCs progenitors

Early lymphoid and myeloid progenitors

The differentiation of HSCs to mature cells of the hematopoietic system is characterized by the progressive loss of developmental options and the generation of intermediate progenitors restricted to certain lineages (13). In the classical model of hematopoiesis, these include common myeloid progenitors (CMPs) that give rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs) (35), and common lymphoid progenitors (CLPs) (36). When lymphoid-primed multipotent progenitors (LMPPs) were discovered, which lacked erythro-megakaryocytic potential but gave rise to all other lineages (37), an alternative model was suggested, in which the erythrocyte/megakaryocytic lineage branches off prior to the division of myeloid and lymphoid lineages. As DC potential is maintained along all of these early hematopoietic progenitors, with the exception of MEPs, this discussion might not be of major relevance for DCs and is thus omitted here. Common surface marker expression, development from thymic progenitors, and transcription factor usage initially led to the interpretation that some subsets of DCs were of lymphoid and others of myeloid origin (38–42). These early conclusions on positive data were later demonstrated to be only one side of the biology as myeloid progenitors were not tested in equivalent assays, and common usage of transcriptional programs or surface marker expression have proven not to be an appropriate criterion to discriminate lineage relation (43–45). We and others could show that both myeloid and lymphoid progenitors give rise to lymphoid organ $CD8\alpha^+$ DCs and $CD11b^+$ DCs (1, 2, 46), as well as pDCs (47), and the terms 'lymphoid' and 'myeloid' DCs should consequently no longer be used in this developmental context (45). The maintenance of DC potential along lymphoid and myeloid committed progenitors is a redundancy, which until now, has not been described for any other cell type in hematopoiesis. The view of true developmental redundancy for DCs is further supported by studies that did not find biological differences in transcriptional programs, surface markers, or functionality in DCs from lymphoid or myeloid progenitor origin *in vivo* (44, 47, 48). The DCs potential of lymphoid and myeloid progenitors was so far investigated in highly permissive assays *in vitro* and in irradiated mice *in vivo*, which can be considered non-physiologic. The distribution of lymphoid- and myeloid-derived DCs in a more physiologic situation, for instance steady-state, non-conditioned animals, remains to be clarified. For now, it seems to be clear that the ratio of DCs from one or the other origin strongly differs between different lymphoid organs, as discussed below.

It is tempting to speculate about the biological significance for the unique redundancy in DC development from lymphoid and myeloid progenitors. A substantial difference between lymphoid and myeloid bone marrow progenitors could be that DC generation of lymphoid progenitors is mainly taking place in organs that are seeded by lymphoid progenitors (as for example in the thymus) (49, 50), whereas myeloid progenitors give rise to DCs in lymphoid and non-lymphoid tissues. Comparing the immune system of species in context of their phylogenic relations, in primitive species like sea urchins and starfish, cells that are similar to that of phagocytes confer resistance to infection by discriminating self from non-self by germline-encoded immune receptors (51). With the appearance of vertebrates, an adaptive immune response developed that is executed by clonally diverse lymphocytes, such as B and T cells. They carry a broad repertoire of immune receptors that can generate immunological memory and more rapidly react to secondary infections with maturated affinities, thus complementing innate mechanisms. With this development, the need for efficient regulation of antigen-presentation evolved. This is mediated by DCs as professional

antigen-presenting cells (APCs), which efficiently connect innate and adaptive immune responses (52–57). DCs might consequently have co-developed with the adaptive immune system from phagocytic cells. The maintenance of DC potential in lymphoid and myeloid pathways might thus ensure the presence and functionality of professional APCs at sites where B or T cells evolve to ensure the efficient selection of functional but self-tolerant mature lymphocytes (52, 58, 59). T cells are the only major hematopoietic lineage that differentiate in high numbers outside the bone marrow. Indeed, the thymus, which is the primary site of T-cell development, is thus far the only organ where lymphoid progenitors contribute to at least half of the DC compartment, while in all other organs investigated, myeloid progenitors seem to be the dominant source, contributing to about 90% of the DC compartment (1, 43–47, 49, 50, 60).

In line with the assumption of developmental redundancy, no functional difference between lymphoid or myeloid progenitor-derived splenic DCs were detected, while substantial functional differences were found comparing DC populations from the thymus and spleen, as demonstrated by Proietto *et al.* (61). Local microenvironments consequently seem to influence DC functionality rather than the developmental origin. For the bone marrow, Kohara *et al.* (62) proposed specific microenvironments for pDCs development. They showed that pDCs localize around CXCL12 (SDF-1)-abundant reticular cells, which build a niche where pDCs can develop in a CXCR4-dependent manner. The developmental origin of DCs in both studies has, however, not been addressed, and a more conclusive picture about different DC progenitors in local microenvironments has yet to be drawn.

DC-restricted progenitors

As pDCs and cDCs were shown to both develop from early common lymphoid and myeloid progenitors and have similar cytokine requirements, the question arose of whether progenitors exist with both pDC and cDC differentiation capacity that are restricted to the DC lineage and do not give rise to any other cells. When we set out to identify such a common DC progenitor, we followed a rational approach, looking at the expression of cytokine receptors relevant for the development of both pDC and cDC in mouse bone marrow, the primary site of hematopoiesis in adult mice. As discussed above, Flt3L is a major, non-redundant cytokine for DC development (25, 26, 63). The receptor for Flt3L, Flt3, a type-III receptor tyrosine kinase, is continuously expressed from progenitor cells to steady-state DCs (26, 64). Mice deficient in Flt3L have reduced numbers of DC progenitors (24), and only the $Flt3^+$ fraction of both myeloid and lymphoid progenitors selectively increased after Flt3L administration, while $Flt3^-$ progenitor frequencies remained constant (64). Consequently, we and others have concluded that DCs development proceeds along a successive line of $Flt3^+$ progenitors and is driven by Flt3L (60, 64, 65, M.A Schmid, D. Kingston, unpublished data). The other candidate cytokine receptors were GM-CSFR and M-CSFR. As we were not able to obtain an antibody that could consistently detect mouse GM-CSFR, we focused on Flt3 and M-CSFR. Within the lineage $(Lin)^-$ fraction of mouse bone marrow cells (i.e. cells not expressing surface markers of any hematopoietic lineage including B cells, T cells, natural killer cells, myeloid cells, DCs, and erythroid progenitors), c-Kit expression, the receptor for stem cell factor, further delineates progenitor populations. Upstream HSCs, multipotent progenitors (MPPs), and myeloid progenitors are found within the c-Kit^{hi} fraction, and we consistently detected multiple lineage offspring within this fraction. The c-Kit intermediate or low fraction (c-Kit^{int}) contains interleukin-7 receptor α (IL7Rα)$^+$ lymphoid and other committed progenitors. In the search for a DC-restricted progenitor, we thus focused on Lin^- c-Kit^{int} cells and found that DC potential was present only within the $Flt3^+$ population. Dissecting lymphoid and DC progenitors within this Lin^-c-$Kit^{int}Flt3^+$ bone marrow fraction using IL7Rα and M-CSFR, we subsequently identified a dividing population of Lin^-c-$Kit^{int}Flt3^+$M-$CSFR^+$IL7Rα^- cells that accounts to approximately 0.1% of bone marrow nucleated cells (66). As a population and on a single-cell level, these progenitors showed efficient generation of pDCs and all lymphoid organ cDC subsets, but they did not produce any other cell types under most permissive conditions *in vitro* and *in vivo*. Therefore, we termed them 'common DC progenitors (CDPs)'. As expected by the cytokine receptors used for their isolation, CDPs were responsive to Flt3L and M-CSF *in vitro*. We demonstrated that CDPs express GM-CSF receptor mRNA by RT-PCR and they produced cDCs in response to GM-CSF *in vitro*. Naik *et al.* (67) in parallel identified a similar pDCs and cDCs-restricted progenitor in an *in vitro* culture system, confirming and extending our *in vivo* findings of a DC-restricted clonogenic common progenitor. *In vivo*, we showed that CDPs produce robust offspring in the bone marrow and spleen, when transferred into non-conditioned hosts, and we were the first to show bone marrow-derived DCs-progenitor engraftment in steady-state lymph nodes (66).

We also addressed the question of whether CDPs are descendants of $Flt3^+$ myeloid and/or lymphoid progenitors. After intra-bone marrow transfer of Lin^-c-Kit^{hi}Sca-1^+ MPPs

and HSCs or $Flt3^+$ myeloid progenitors, both generated CDPs by phenotype (66). We could not detect substantial numbers of CDPs from $Flt3^+$ lymphoid progenitors in steady-state. Therefore, whether DCs from lymphoid progenitors develop via CDPs as an intermediate state or follow an alternative developmental path could not be conclusively answered.

While we were working on the identification and isolation of CDPs as the common progenitor for pDCs and cDCs, Fogg *et al.* (68) characterized macrophage dendritic progenitors (MDPs) as clonogenic bone marrow progenitors common for macrophages, monocytes, and cDCs but not pDCs using CX_3CR1-eGFP reporter mice. At the time, it was difficult to integrate both MDPs and CDPs into a cohesive concept of DCs development, because MDPs could only be isolated from CX_3CR1-eGFP reporter mice, which are not commonly available. Further studies showed that MDPs can also give rise to pDCs and are the direct progenitors of CDPs (69) (Fig. 1). Further downstream of CDPs, 'pre-cDCs', which generate all cDCs

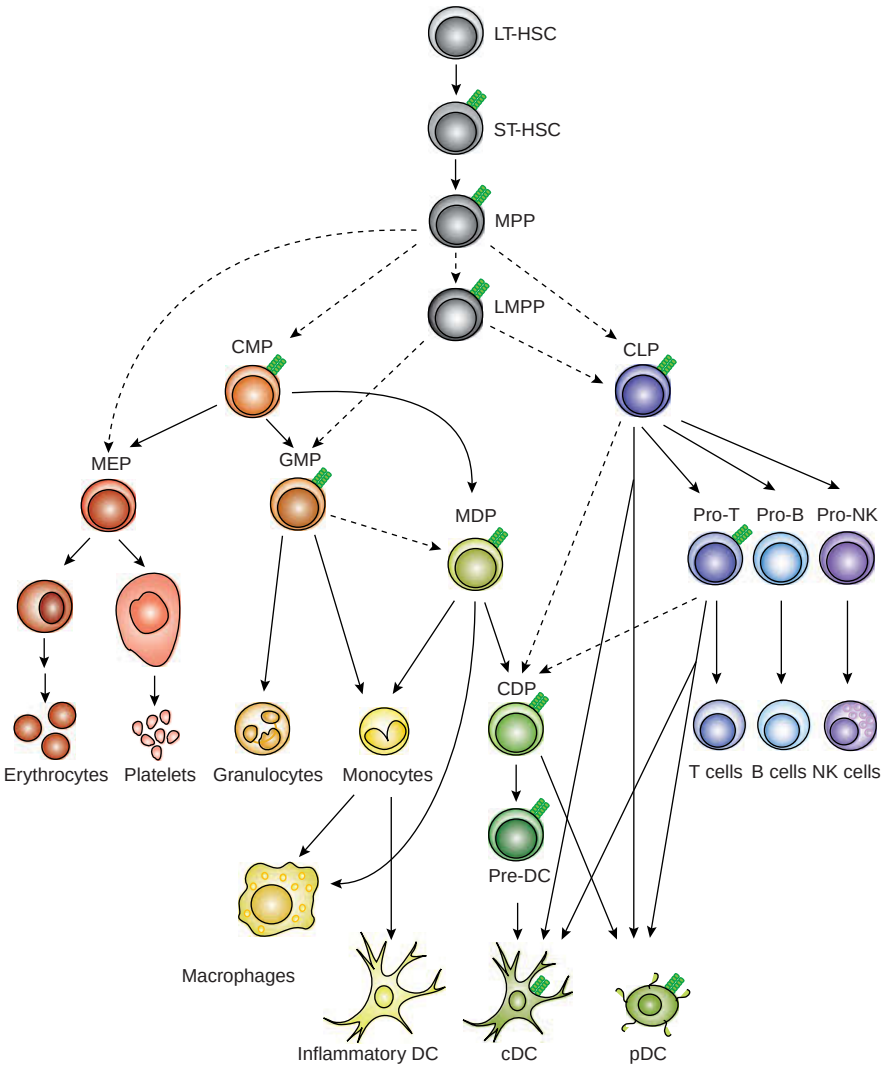

Fig. 1. Hematopoietic tree for dendritic cell development. Hematopoiesis is initiated in the bone marrow by long-term hematopoietic stem cells (LT-HSCs), which continuously self-renew but also give rise to proliferating short-term HSCs that then further lose their self-renewal potential and commit via multipotent progenitors (MPPs) to mature cells. Further downstream, lineage differentiation potential branches into progenitors committed to myeloid cells, common myeloid progenitors (CMPs), or lymphoid cells, common lymphoid progenitors (CLPs). CMPs then further differentiate to megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). Alternatively, MPPs develop to recently described lymphoid-primed multipotent progenitors (LMPPs) that have lost ME potential but carry DC, myeloid, and lymphoid developmental options. DC potential is retained in all CMPs, CLPs, and LMPPs and is restricted to progenitors expressing Flt3 (green receptor on cells). Macrophage-DC progenitors (MDPs) give rise to monocytes, macrophages, classical DCs (cDCs), and plasmacytoid DCs (pDCs). MDPs-derived monocytes can further differentiate into inflammatory DCs. MDPs lie upstream of the common DC progenitors (CDPs), which are DC-restricted, giving rise to pDCs and, via pre-DCs, to cDCs. Pro-T, T-cell progenitor; Pro-B, B-cell progenitor; Pro-NK, natural killer cell progenitor. Solid arrows show demonstrated pathways; dotted arrows show suggested pathways that have not been formally proven.

subsets but no pDCs, monocytes, or macrophages (22), were later integrated into the scheme as the direct descendants of CDPs after pDCs potential is lost (69). Recent data suggest that CDPs via pre-cDCs can give rise to some subsets of intestinal *lamina propria* DCs (70, 71) and potentially other non-lymphoid tissue DCs, further extending the *in vivo* relevance of the intermediary CDPs state. As the term 'cDC' is generally used for lymphoid tissue-resident DCs, we in the following use the term 'pre-DC' as the progenitor of lymphoid tissue 'cDC' and non-lymphoid tissue 'DCs'. As MDPs are considered to be of the myeloid lineage, this might further support the view that CDPs and subsequently pre-DC are an intermediate DCs progenitor within the myeloid branch of the hematopoietic tree. Whether DC development from lymphoid progenitors converges at a certain point or whether they develop independently from each other remains to be shown.

Incorporating the most recent findings, the early hypothesis that Flt3L continuously drives DC development along $Flt3^+$ progenitors (64) can now easily be included in the current view of DCs development (*Fig. 1*): Long-term HSCs (LT-HSCs) give rise to $Flt3^+$ short-term HSCs (ST-HSCs) and subsequently $Flt3^+$ MPPs that further differentiate to either $Flt3^+$ LMPPs (37) and $Flt3^-$ erythrocyte–megakaryocyte progenitors (MEPs), or $Flt3^+$ common myeloid progenitors (CMPs) and $Flt3^+$ CLPs (36). The sub-fractions of CMPs and CLPs maintaining DC potential express Flt3 (37, 64, 72), whereas MEPs lacking DC developmental options do not. CMPs give rise to GMPs and after granulocyte progenitors have branched off, likely, to MDPs (68) – although the GMP to MDP step remains to be formally proven. Flt3 expression is retained in the MDPs population carrying pDCs, cDCs, monocytes and macrophage potential, followed by the continuous expression of Flt3 in the downstream CDPs and pre-DCs (66, 69).

Development from upstream progenitors carrying broad lineage potential, down to DC-restricted CDPs, takes place within the bone marrow. Pre-DCs, which were initially characterized in the spleen (22), were later shown to be present in bone marrow, blood, and lymph nodes giving rise to lymphoid tissue cDCs (69) and some subsets of non-lymphoid tissue DCs of the gut (70, 71). As a main pathway, it consequently seems likely that $CD11c^-$ CDPs in steady-state are mostly bone marrow-resident cells that give rise to pDCs and pre-DCs, which then circulate to seed lymphoid organs and non-lymphoid tissues and develop into steady-state DCs. Furthermore, we identified progenitors with CDP-phenotype but expressing CD11c in the spleen, which produce pDC and DC offspring (66). This observation opens the possibility that some CDP, under certain circumstances, might leave the bone marrow and enter circulation. Which of the DCs progenitors can exit the bone marrow and in steady-state and upon specific demand seed different organs needs to be determined in future research.

Integrating the above concepts into cytokine microenvironments *in vivo*, we could show that MDPs and CDPs are dependent on Flt3L for their maintenance and/or survival in the bone marrow (26). Furthermore, both progenitors express the receptors for Flt3L and GM-CSF, and the combined absence of the two cytokines in double deficient mice resulted in a substantial reduction of DCs-progenitor cell numbers compared with Flt3L single-deficient mice. This observation uncovers a synergistic role of Flt3L and GM-CSF in the maintenance of DC-progenitor cells. In line with these results, CLPs, which also give rise to DCs and express Flt3, are significantly reduced in Flt3L-deficient mice (26, 63). Furthermore, recent studies further suggest differential roles of Flt3L, GM-CSF, and M-CSF for the development or maintenance of different subsets of non-lymphoid tissue DCs from CDPs and pre-DCs, or monocytes (26, 70, 71). Further investigations will determine cytokine requirements for lymphoid- and non-lymphoid tissue DCs in more detail. Thus, the interaction of cytokine receptor-expressing progenitors and the surrounding environment are critical factors regulating both early DC-progenitor numbers and mature DC homeostasis.

Instructive DCs lineage commitment

It is a long-standing question whether lineage commitment from HSCs is a stochastic process determined by intrinsic differentiation programs (intrinsic, stochastic lineage commitment) or whether cytokines and other extrinsic factors instruct HSCs to differentiate to MPPs and subsequently to lineage-restricted precursors (extrinsic lineage instruction) (73). In the model of stochastic lineage commitment, independent of external signals, progenitors of all lineages would form at any time. Regulation in this model would occur by selection of these stochastically formed progenitors that receive a positive signal, whereas others would undergo apoptosis. It now becomes apparent that HSC promiscuously express various genes normally found in more differentiated cells, and this expression allows them to receive signals that can skew their differentiation to a particular lineage. Recent studies give strong evidence that cytokines instruct uncommitted progenitors to commit to a particular lineage. Rieger *et al.* (74) showed in single-cell time-lapse imaging how M- or G-CSF can instruct the lineage choice of GMPs to almost exclusively (approximately

90%) differentiate into either the macrophage or the granulocyte lineage. The general absence of cell death observed makes it unlikely that offspring was generated by positive selection of stochastically pre-formed progenitors.

Cytokine receptor expression and cytokine availability

In the extrinsic model, successive instructive signals drive MPPs to further differentiate to downstream, lineage-committed progenitors. In this context, what are the factors that determine whether a progenitor is restricted to certain lineages and has lost the capacity to differentiate to others? The expression of a specific set of lineage-related cytokine receptors would be the most substantial prerequisite to receive lineage instructive signals and generate offspring of that lineage. In the recent study by Rieger *et al.* (74), which gives strong evidence that cytokines instruct lineage choice, *in vitro* models were used in which the respective cytokine was present in saturating concentrations. In more physiologic situations, cytokine concentrations will vary locally and will be particularly high in specialized microenvironments or niches. In addition, the level of cytokine receptor expression will vary between different progenitors and might itself be regulated by extrinsic factors. Consequently, the combination of cytokine concentration and receptor expression determines whether a progenitor receives a sufficiently high signal to differentiate to a particular lineage. This can be achieved in two situations: First, if a high cytokine concentration is available to progenitors (*Fig. 2A*, left), they will develop irrespective of high or low expression of the respective receptor. This abundance of cytokine will occur at sites or niches where commitment and differentiation typically takes place. Second, if only small amounts of cytokine are available (*Fig. 2A*, right), progenitors with high receptor expression will still receive sufficient amounts of positive signal to differentiate and give rise to offspring of the corresponding lineage. However, progenitors with low receptor expression will undergo apoptosis or develop into alternative lineages if they are responsive to alternative signals.

More than one cytokine will act in concert on a cell *in vivo*. Progenitors, which carry the potential to differentiate into two lineages, will express cytokine receptors relevant for both of

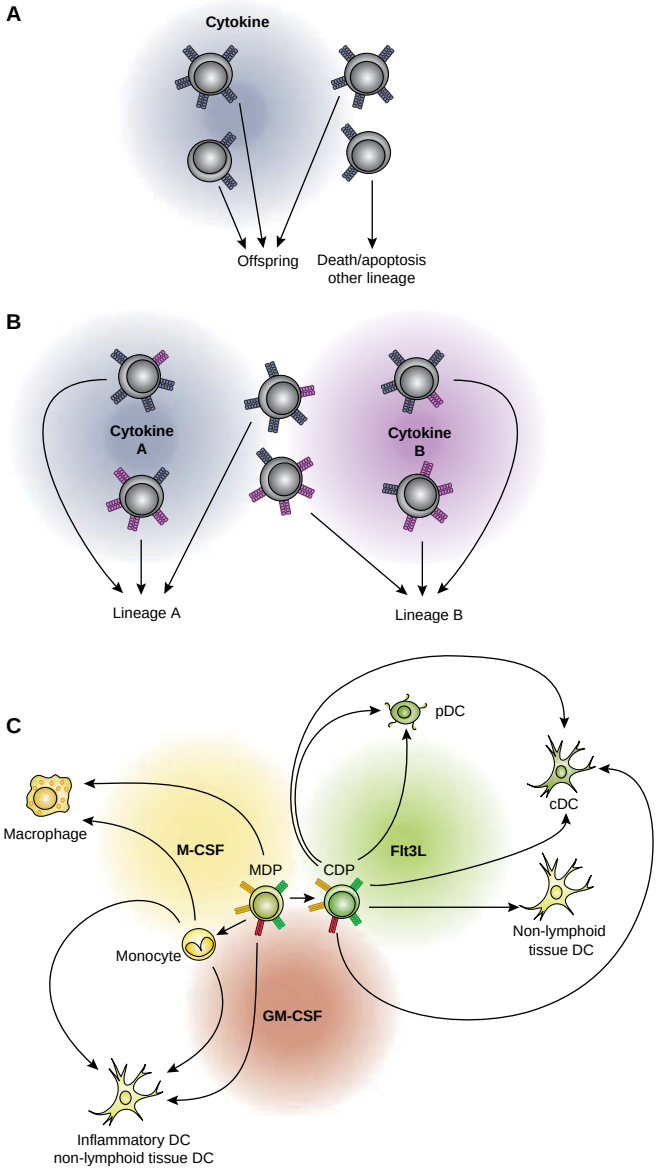

***Fig. 2.* Hypothesis for cytokine-induced differentiation of progenitors.** (A) One cytokine, one receptor scenario: If two progenitors, one with high receptor expression, the other with low receptor expression, are located near high concentrations of the respective cytokine, both cells will acquire sufficient signal to differentiate and give rise to offspring (left). If both cells are exposed to low concentrations of the cytokine, only the cell with high receptor expression will receive sufficient signaling to differentiate, while the cell with low receptor expression will not and will either undergo apoptosis or differentiate into another lineage (right) if it receives signaling through an alternate receptor (not pictured). (B) Multiple cytokines/receptors scenario. As in (A), if two progenitors, one being receptor A^{high} receptor B^{low}, the other receptor A^{low} receptor B^{high}, are exposed to high concentrations of cytokine A, both will differentiate into lineage A (left), or conversely, if both progenitors are near very high levels of cytokine B, both cells will give rise to cells from lineage B (right). If both progenitors are exposed to only low concentrations of both cytokine A and cytokine B, the cell expressing receptor A^{high} receptor B^{low} will preferentially differentiate into lineage A, while the A^{low} receptor B^{high} cell will differentiate into lineage B. (C) macrophage dendritic progenitors (MDPs) and common DCs-progenitors commitment is dependent on cytokine exposure. MDPs and CDPs express the receptors for fms-related tyrosine kinase 3 ligand (Flt3L), granulocyte–macrophage colony-stimulating factor (GM-CSF), and M-CSF. MDPs have the potential to differentiate into macrophages, monocytes, and inflammatory DCs, and via CDPs to cDCs and pDCs. Different microenvironments with variations in the combination and concentration of the three cytokines influence the lineage commitment and differentiation of MDPs and CDPs to mature cells.

these. In Fig. 2B, a cell expressing high levels of the receptor for cytokine A and low levels of the receptor for cytokine B (upper cells) and a cell expressing low receptor A and high receptor B (lower cells) are shown to be situated in an environment with overlapping concentrations of these cytokines. When both cells are located in a milieu with high concentrations of cytokine A (Fig. 2B, left), they would differentiate to lineage A, whereas they would both give offspring to cells of lineage B in a milieu with high concentrations of cytokine B (Fig. 2B, right). In the region with overlapping amounts of cytokines A and B (Fig. 2B, center), cells with high expression of receptor A would differentiate to the A lineage, whereas cells with high expression of receptor B would generate cells of lineage B.

In our earlier work, we proposed that DC development would be demand-regulated and cytokine-driven (75, 76). We now suggest a signal strength-dependent model in which the signals that lead to DC development are integrated from cytokine receptor expression, downstream signals, and the availability of cytokines in specific niches. MDPs, for example (68) expressing receptors for M-CSF, Flt3L, and GM-CSF (69), can differentiate into monocytes, macrophages, pDCs, and cDCs. In Fig. 2C, different theoretical outcomes are illustrated when MDPs are exposed to various overlapping concentrations of M-CSF, Flt3L, or GM-CSF in different microenvironments. MDPs that are exposed to high concentrations of M-CSF might be instructed to generate monocytes and macrophages, whereas in high concentrations of Flt3L or in combination with M-CSF, they might give rise to CDPs (69) and subsequently pDC and cDC progeny (66, 67). GM-CSF was shown to drive the generation of inflammatory DCs offspring from monocytes (17). From CDPs, which do not carry monocyte potential, GM-CSF leads to the exclusive generation of cDCs, as well in combination with Flt3L and/or M-CSF (M.A. Schmid, unpublished data).

Bringing progenitors with DC potential into a successive order, it becomes apparent that the levels of cytokine receptor expression designate lineage potential (Fig. 3). Early progenitors, such as LT-HSCs and LMPPs, express high levels of c-Kit, whereas high expression of Flt3 first occurs at the LMPPs level and is maintained in CLPs. CLPs generate all cells of the lymphoid lineage, are positive for IL7Rα, and in line with their high Flt3 expression, maintain DC potential (2, 47, 75). Further differentiation along the lymphoid line and full B-cell commitment is accompanied with further upregulation of IL7Rα but loss of Flt3 expression and accordingly loss of DC potential. For the myeloid branch of hematopoiesis, MDPs express intermediate to high levels of c-Kit, Flt3, and M-CSFR, and are negative for IL7Rα. Further differentiation into CDPs and loss of monocyte/macrophage potential is accompanied by a downregulation of c-Kit, while still retaining developmental potential for pDCs and cDCs. In recent experiments, we found that within the originally defined CDP population (66), lin$^-$c-Kit^{int}Flt3$^+$IL7Rα$^-$M-CSFR^{high} cells are already cDC committed, whereas true bi-potential for cDCs and pDCs lies within the M-CSFR^{low+} portion of this progenitor fraction (M.A Schmid, unpublished results). These results suggest that commitment to the cDC lineage could occur by further upregulation of M-CSFR. How this M-CSFR^{hi} bone marrow cDC progenitor can be integrated with the previously defined pre-DC (22, 69) remains to be determined.

In this context, a set of cytokine receptor expression predefines the lineage potential of hematopoietic progenitors and their responsiveness to certain cytokines. It therefore seems likely that MPPs express different cytokine receptors at moderate levels, and lineage restriction occurs when lineage-specific cytokine receptors are upregulated and expression of cytokine receptors for alternative lineages are lost. Upon urgent need, such as during severe infection or when damage to the hematopoietic system occurs by environmental toxins (or irradiation), accordant lineage commitment is regulated via the availability of cytokines. A massive increase of a cytokine can consequently even drive progenitors with low receptor expression to the required lineage.

The local regulation of cytokine production during physiological stress

Given that Flt3L is a key cytokine in DC differentiation, it is important to understand the mechanisms leading to the regulation of Flt3 and Flt3L expression during steady-state and inflammation. Flt3L is produced by both hematopoietic and non-hematopoietic compartments in mice (S. Boddupalli, unpublished results). This is performed mostly by T cells, stromal cells, and endothelial cells, and Flt3L is readily detectable in the serum in steady-state (77–80). Flt3L has two splice variants, a membrane-bound form and a soluble form (78, 81, 82). In situations leading to hematopoietic stress, such as irradiation when substantial cell loss and inflammation occurs, serum Flt3L levels increase dramatically (83, 84; M.A. Schmid, unpublished results), whereas GM-CSF levels might be increased locally. Our own preliminary data suggest that during inflammation, mRNA expression of Flt3L in stromal cells remains constant, while mRNA for GM-CSF is rapidly upregulated. Lymphocytes upon activation rapidly proliferate and secrete Flt3L (D. Kingston and S. Boddupalli, unpublished results). It is tempting to speculate that

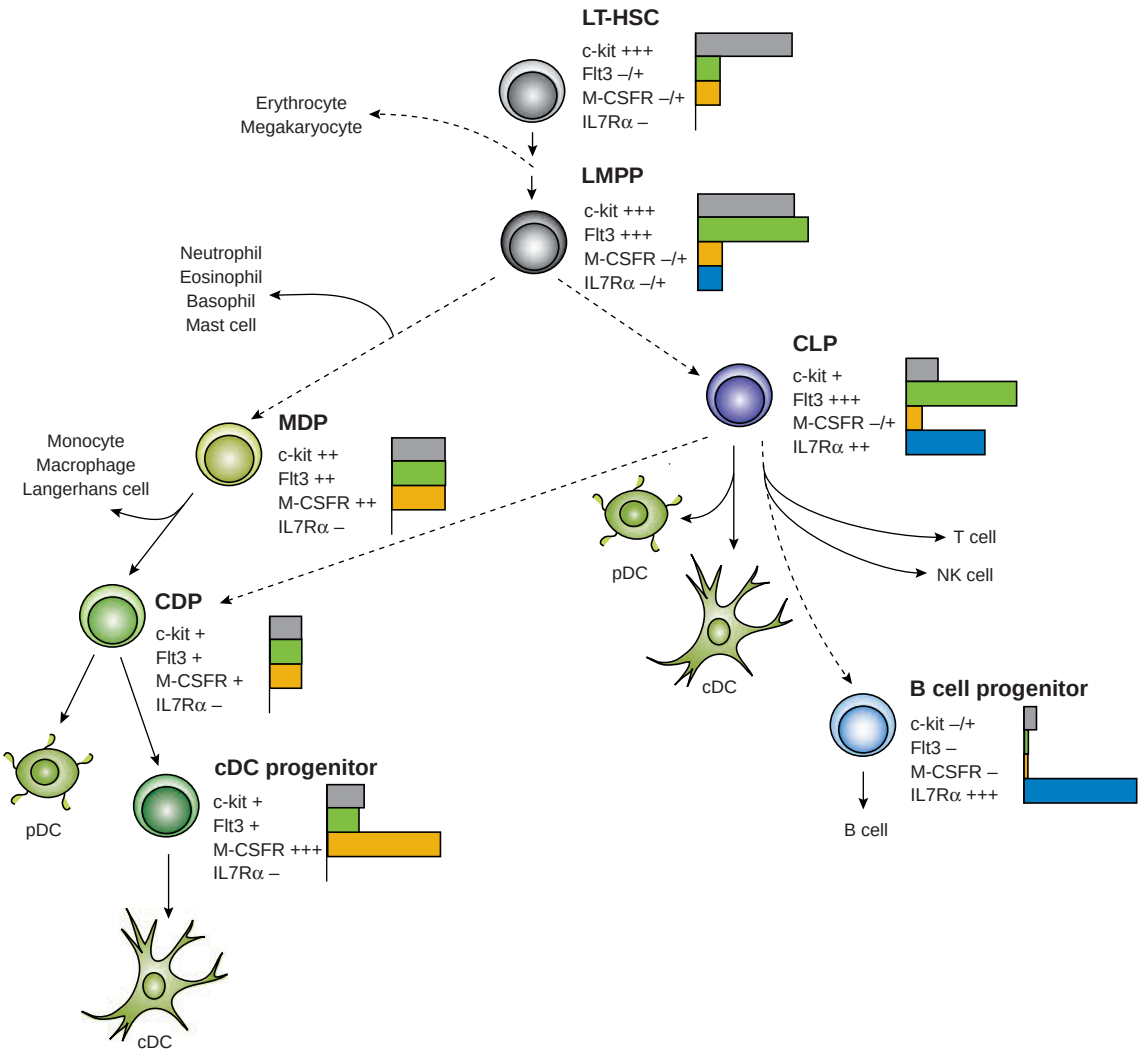

***Fig. 3.* Cytokine receptor expression along dendritic cell (DC) differentiation pathways.** The level of receptor expression for c-Kit (gray), Flt3 (green), M-CSFR (yellow), and interleukin-7 receptor α (IL7Rα) (blue), as determined by surface staining and flow cytometric analysis, is shown for successive progenitors developing into DCs. Differentiation of progenitors coincides with the downregulation of c-Kit expression. Lymphoid progenitors with DC potential retain high levels of Flt3 and low levels of M-CSFR expression. Upon differentiation to the B-cell lineage and loss of DC potential, B-cell progenitors further upregulate IL7Rα and lose expression of Flt3. Myeloid progenitors have lost IL7Rα expression, while retaining M-CSFR and Flt3 expression. Further differentiation to cDCs is accompanied by upregulation of M-CSFR expression. Thus, intermediate multi-lineage progenitors express intermediate levels of receptors for cytokines linked to several different lineages. Lineage commitment consequently occurs when certain cytokine receptors are upregulated, and receptors for alternative lineage cytokines are lost. Bar-length and + and – indicate levels of cytokine receptor expression.

Flt3L produced locally by proliferating, recently activated T cells supports DC replenishment from DC progenitors in lymphoid tissues. This could ensure sufficient replacement of dying APCs during an ongoing immune reaction. Furthermore, stromal cells could also contribute to increases in Flt3L after inflammation through post-translational modifications of Flt3L and release of intracellular protein stores (83). In addition to the increase of Flt3L levels, Flt3 signals are also amplified intrinsically: Flt3 signaling leads to the upregulation of Flt3 mRNA transcription and the initiation of a self-sustained positive feedback loop, which drives DC differentiation from $Flt3^+$ progenitors (75).

The correlation of Flt3L levels, Flt3 signaling, and DC numbers have been addressed in different experimental setups. Pharmacologic disruption of Flt3 signaling using the small molecule receptor tyrosine kinase inhibitor SU11657 inhibited DC generation from Flt3L-supplemented whole bone marrow cultures (85). Treatment of mice with SU11657 or CEP-701 resulted in a decrease in the numbers of cDCs and pDCs *in vivo*, mirroring the phenotype observed in Flt3L-deficient mice (85, 86). These observations show that DC numbers decrease once Flt3 signals cease. The system tries to counterbalance these effects by increased Flt3L production, as Flt3L serum levels were highly increased after Flt3 inhibitor

treatment (85) and in Flt3 receptor-deficient animals (S. Boddupalli and D. Kingston, unpublished results). After discontinued administration of the Flt3 inhibitors, DC levels increased to normal levels, while at the same time, Flt3L serum levels decreased back to homeostatic concentrations. In addition, the specific depletion of cDC using transgenic reporter mice expressing the diphtheria toxin receptor under control of the CD11c promoter led to elevated levels of Flt3L (57). It thus becomes clear that low Flt3L levels or diminished Flt3 signaling leads to low numbers of DCs. In contrast, low numbers of DCs or diminished Flt3 signaling leads to high levels of Flt3L. This observation further shows that Flt3L levels are tightly regulated and closely connected with DC numbers, which could be a result of changes in Flt3L production and consumption. Our own results suggest that Flt3 receptor expression on steady-state DCs might act as a sink for Flt3L, thus regulating Flt3L levels and subsequent maintenance of DC homeostasis (S. Boddupalli, unpublished results).

DCs numbers seem to inversely correlate with the numbers of regulatory T cells (Tregs), as demonstrated recently by the increase in DC numbers after Foxp3$^+$ Treg depletion (69, 87, 88). Moreover, the expansion of DCs after Treg depletion is dependent on Flt3 expression and signaling (69). Although an inverse correlation between Treg and DCs numbers is observed after Treg depletion, administration of Flt3L expands DCs and Tregs (89), while the converse is observed in Flt3L-deficient mice, where both DC and Treg levels are reduced (87). These results suggest an intricate regulatory mechanism, involving Tregs and most likely being Flt3L-dependent, that maintains homeostatic DC numbers to balance immune activation with peripheral tolerance. The discrepancies between the effects seen after Treg depletion and Flt3L-administration or deficiency remain to be integrated into physiologic settings (90).

Transcription factors and the intrinsic sensitivity to cytokine signals

Ligation of cytokine receptors on progenitor cells leads to the activation of downstream signals and transcription factors (13). Several transcription factors are important for the development of different DC subsets, as reviewed elsewhere (45). The importance of both transcription factors and cytokine signals for lineage determination was emphasized in a recent study by Sarrazin *et al.* (91). The authors suggest that the transcription factor MafB restricts the sensitivity of HSCs to respond to M-CSF signaling and thus plays an important role in maintaining the balanced lineage potential of HSCs by restricting divisions that lead to myeloid commitment. This commitment to the myeloid lineage occurs when one of the daughter cells, after asymmetric division, is PU.1$^+$. Consequently, by decreasing the sensitivity to M-CSFR signals, MafB limits the number of downstream progenitors that are dominated by PU.1 signals and thus are restricted to the myeloid lineage. In addition to the level of cytokine receptors expressed by a progenitor cell that enables it to receive a lineage instructive cytokine signal, downstream transcription factors can modulate the intrinsic sensitivity to cytokine signals. Furthermore, transcription factors can directly block alternative transcriptional programs, skewing progenitors to a certain lineage (92).

Investigating the role of Flt3 receptor and its downstream signals in DC development, we showed that activation of the Flt3 signal transduction cascade rescues and enhances pDC and cDC development (75): Ectopic expression of Stat3 in Flt3$^+$ progenitors led to further upregulation of Flt3 receptor expression and enhanced DC offspring. This finding suggests that Flt3 signals lead to a self-sustained positive feedback loop that drives DC differentiation. Furthermore, in Flt3$^-$ MEPs, which under physiologic conditions are restricted to megakaryocyte/erythrocyte development, enforced expression of Flt3 or the downstream transcription factors Stat3 and PU.1, instructed differentiation into pDCs, cDCs, and myelomonocytic cells. This further strengthens the hypothesis that cytokine receptor expression and downstream signals instruct lineage choice.

It has long been observed that bone marrow progenitors cultured in Flt3L differentiate to pDCs and cDCs, whereas cultures with GM-CSF alone or in combination with other cytokines lead to the exclusive production of cDCs and inhibited development of pDCs (19). A recent study identified signal transducer and activator of transcription 5 (Stat5) as the downstream transcription factor activated upon GM-CSFR engagement that directly inhibits pDC development by suppressing the transcription factor IFNs regulatory factor 8 (IRF8) through direct binding to its promoter (93, 94). However, in GM-CSF-deficient mice, no effects on the number of pDCs could be observed in any of the tissues analyzed (26). Consequently, at least in steady-state, GM-CSF does not deliver an inhibitory signal for pDC development *in vivo* (26).

Conclusions and future directions

Distinct levels of regulation lead to the differentiation of sufficient numbers of DCs in diverse organs throughout the body

in steady-state and upon urgent need during inflammation. Microenvironments are critical in providing a specialized cytokine milieu allowing progenitors that express a particular set of cytokine receptors to generate respective DC subsets. In future research, it will be important to identify and characterize the spatial niches for DC development and to determine the respective localization of intermediate progenitors. This identification will help to clarify which steps of DC development mainly occur in specific niches inside the bone marrow and which progenitors develop in microenvironments in peripheral lymphoid organs or non-lymphoid tissues. An important question is how the migration and subsequent localization of progenitors in microenvironments for DC generation occurs. In ongoing investigations, we identified substantial differences in chemokine receptor expression on DC progenitors in steady-state and during inflammation and their specific guidance to inflamed lymphoid organs (M.A. Schmid, unpublished data). Future research will show which factors influence the specific localization and migratory behavior of DC progenitors.

Identifying the regulation of DC homeostasis is essential to harness DCs function for therapeutic concepts in infectious diseases, allergy, autoimmunity, and tumor immunity in humans (95). It will consequently be critical to translate the accumulating knowledge in mice to the human system and determine the conserved concepts between mouse and human DC development. Lymphoid and myeloid common progenitors have been identified in humans (96), which, as in mice, give rise to pDCs and cDCs (97, 98), and DCs from both species do not show substantial differences in their gene expression patterns (48). Furthermore, human and mouse DCs development have similar cytokine requirements. In future research, hematopoietic system humanized mice might be a valuable tool to verify data obtained from mice and human cells *in vitro*, in an *in vivo* setting (99–101).

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Bone marrow stromal cells sense TLR4 agonists and subsequently enhance myelopoiesis

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ABSTRACT

Hemato-lymphopoiesis is tightly regulated by growth factors that act on stem and progenitor cells in the bone marrow. The mechanisms that guide myeloid cell generation upon specific demand have not been defined. Here, we demonstrate that human bone marrow stromal cells express TLR4 and that receptor stimulation leads to production of factors supporting early hematopoietic progenitors and myeloid cell differentiation. Also, we show that non-hematopoietic cell expressed TLR4 is both essential and sufficient for LPS induced myelopoietic responses in mouse bone marrow *in vivo*. These findings reveal a potent regulatory mechanism how systemically available pathogen signals are translated into demand-adapted production of innate immune effector cells at primary hematopoietic sites.

INTRODUCTION

A small fraction of bone marrow located hematopoietic stem cells (HSCs) support via both self-renewal and stepwise commitment to hierarchically organized lineage committed progenitors the development of all hematopoietic cells throughout the lifetime of an individual[1]. This process is tightly regulated via remote or bone marrow produced growth factors, acting on respective receptor expressing cells, allowing steady-state homeostasis, as well as enhanced production of cell populations upon specific demand. Growth factor availability is determined by constitutive and inducible production, as well as by consumption (reviewed for example in[2]). Red blood cell production is for example supported by constitutive kidney and liver produced erythropoietin, and hypoxia caused by low red blood cells or insufficient oxygenation of these is sensed by interstitial cells of the renal cortex, leading to induced renal erythropoietin production and subsequently enhanced erythropoiesis until a steady-state is achieved. In contrast, thrombocyte production is at large regulated via constitutive production of thrombopoietin that is sequestered by platelets, with low platelet counts leading to higher thrombopoietin availability and megakaryocyte stimulation.

Bone marrow granulo-monocytic cell differentiation is supported by multiple cytokines, for example by broadly acting IL-3, IL-6 and IL-11, and more specifically acting granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF),

that are produced by hematopoietic cells and non-hematopoietic tissues at multiple sites throughout the body[3-5]. In inflammatory conditions like sepsis, where innate immune system cells are in high demand, cytokines such as G-CSF are elevated in serum, bone marrow myelopoiesis is enhanced, and myeloid colony-forming progenitor cells and granulocytes increase in circulation[6-9]. Although this is common clinical knowledge, the mechanisms that regulate the availability of myelo-monocytic acting cytokines in bone marrow upon increased demand, so called “emergency-granulopoiesis”, are not well defined.

Rapid immune response to infection depends on the interaction of pathogen-associated molecular patterns (PAMPs) with their specialized pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) belong to the PRR family and sense conserved microbial products as well as some endogenous ligands[10, 11]. TLR expression and function has been mostly studied on innate and adaptive immune system effector cells; however, TLRs are also expressed on some non-hematopoietic tissues and are involved in regulating immune responses and tissue regeneration[12]. TLR4 that binds lipopolysaccharide (LPS) is for example expressed on lung endothelium and respective stimulation is critically involved in LPS induced lung neutrophil sequestration[13, 14]; also, TLR4 is expressed on bladder epithelial cells, and respective stimulation is involved in control of gram-negative mucosal infection[15].

The bone marrow microenvironment provides the home for HSC maintenance and hematopoietic differentiation. It consists of multiple cell types including vasculature, bone, fat, fibroblast-like stromal cells, and bone marrow stromal cells (BMSCs), that are in intimate contact with hematopoietic cells[16]. BMSCs can be isolated *in vitro* by plastic adherence[17] and, under appropriate culture conditions, a subset of these cells can be differentiated into multiple mesenchymal tissues such as bone, fat or muscle[18-20]. Thus, BMSCs were termed mesenchymal stem cells, or more recently, multipotent mesenchymal stromal cells (MSCs)[18-20]. However, as questions regarding the heterogeneity of these cell populations are unsolved, we here will call bone marrow derived, plastic adhering cells BMSCs.

BMSCs were demonstrated to have potent immunosuppressive functions and have been used in clinical settings to reduce graft versus host disease[21, 22]. Furthermore, it was recently demonstrated that intravenous infusion of BMSCs in a mouse model of sepsis can induce monocytes to produce IL-10 and subsequently attenuate death rates[23]. However, as BMSCs are part of the sessile bone marrow

microenvironment, we assume that their primary physiologic role is likely to support hematopoiesis locally in steady-state and upon diverse hematopoietic challenges[16]. To serve this task according to needs during generalized infections, we hypothesized that BMSCs express TLRs and are capable to respond to TLR agonists by changing their hematopoiesis supporting growth factor production.

RESULTS

Human BMSCs express TLRs

Primary human BMSCs were obtained from total bone marrow cells by plastic adherence[20]. Cultured BMSCs fulfilled MSC defining properties according to current consensus[18, 19] (Fig. S1 A, B). TLR mRNA expression in BMSCs was evaluated in comparison to human peripheral blood dendritic cells (DCs; BDCA-1$^+$, CD14$^-$, CD19$^-$) and natural type I interferon producing cells (IPCs, BDCA-4$^+$, CD14$^-$, CD19$^-$), both known to express distinct functional TLRs[24, 25]. Analysis of mRNA expression in BMSCs revealed that *Tlr3* and *Tlr4* were expressed about 2 log higher, whereas *Tlr1, Tlr5* and *Tlr6* were expressed at slightly higher levels than in DCs (Fig. 1 A). *Tlr9* was expressed at high levels in IPCs but at low levels in BMSCs, i.e. at comparable levels to DCs which are known to not carry the functional TLR9 protein[24, 25]. Expression of *Tlr2*, *Tlr7*, *Tlr8* and *Tlr10* was not detectable in BMSCs within the sensitivity of the assay (Fig. 1 A). mRNA expression of MD-2, an adaptor protein required for TLR4 signaling[26], was about 2 log higher in BMSCs than in DCs and IPCs (Fig. S2 A). In line with the data from the primary human BMSCs analyzed, we observed similar expression of *Tlr3*, *Tlr4*, *Tlr9,* and *Md-2* in a human TERT-immortalized bone marrow MSC line[27] (Fig. S2 A, B). Thus, primary human BMSCs and human TERT-immortalized MSCs express high levels of *Tlr4* and the adapter protein *Md-2* which potentially renders them capable of directly responding to TLR4 agonists.

TLR4 stimulation on human BMSCs induces production and release of functional myeloid cell differentiation supporting cytokines *in vitro.*

Stimulation of human BMSCs with the TLR4-agonist LPS induced mRNA expression of at baseline undetectable *G-csf* and *Gm-csf,* and increased constitutive expression *M-csf*, *Il-6, and Il-11,* as well as expression of early hematopoietic acting cytokines *thrombopoietin* (*Tpo*) and *flt3-ligand* (*Flt3l*) (Fig. 1 B). In line with mRNA expression results, LPS induced production of G-CSF and GM-CSF protein, and significantly

enhanced constitutive secretion of M-CSF, IL-6, and IL-11 (Fig. 1 C). SCF, SDF-1, and IL-7 secretion was not significantly changed, and, although increased at mRNA levels, TPO and Flt3L were not measurable in supernatants of LPS stimulated BMSCs (Fig. 1 B, C and Fig. S2 C and data not shown). In accordance with low *Tlr9* mRNA expression, CpG did not induce nor enhance production of G-CSF, M-CSF, GM-CSF, and IL-7 (Fig. S2 C). Similar as in primary human BMSCs, increases of G-CSF, M-CSF, and GM-CSF were observed in supernatants of LPS stimulated human TERT-immortalized MSC (Fig. S2 D).

We next tested the biological activity of secreted cytokines in myeloid colony-formation unit (CFU) assays using human cord blood (CB) $CD34^+$ hematopoietic stem and progenitor cells[28]. As supernatants of human BMSC or TERT-immortalized MSC cultures contained low or non-detectable levels of early acting cytokines SCF, TPO, and Flt3L necessary for CFU assays, these cytokines were supplied in addition. Constitutively BMSC- and TERT-immortalized MSCs expressed cytokines supported the formation of myeloid colonies, while in contrast to a fully cytokine supported CFU control, mixed or pure erythroid colonies were not detected (Fig. 1 D and Fig. S3 A). CFU activity was up to 2.5-fold higher when supernatants of TLR4-stimulated BMSCs and TERT-immortalized MSCs were used, an effect that could be partly mimicked by adding equivalent amounts of recombinant G-CSF, GM-CSF and M-CSF to supernatants of un-stimulated BMSCs, while direct LPS addition to non-stimulated BMSC media or media alone did not change CFU activity.

We thus conclude that TLR4 stimulation on human primary BMSCs and TERT-immortalized MSCs from bone marrow induces *de novo,* and enhances constitutive production and release of functional myeloid cell differentiation supporting cytokines that are capable to stimulate quantitative increase of CFU-GM/G/M from a given number of human hematopoietic stem and progenitor cells. This process is mediated in part by stromal cell secreted G-CSF, GM-CSF, and M-CSF.

TLR4 stimulation on human BMSCs enhances hematopoietic progenitor cell maintenance *in vitro.*

Given the constitutive secretion of SCF and the transcriptional increase in the early acting hematopoietic cytokines TPO and Flt3L upon TLR4 stimulation without protein release in supernatants, and the described action of membrane bound SCF and Flt3L[16, 29], we evaluated the capacity of human BMSCs to maintain immature CB

$CD34^+$ cells in co-cultures with or without addition of LPS. Within 12 days of co-culture total hematopoietic cell numbers increased (2-3 population doublings), but cell number increase was higher in BMSC-free cultures supplemented with SCF, TPO and Flt3L only (5-6 population doublings) (Fig. 2 A). No difference in total cell numbers was seen with respect to TLR4 activation in either type of culture (Fig. 2 A). However, LPS addition to BMSC and CB $CD34^+$ cell co-cultures led to an about two-fold higher percentage of recovered $CD34^+$ cells (32.36±3.25% of total hematopoietic cells) as compared to BMSC and CB $CD34^+$ cell co-cultures without LPS (15.23±2.37%). No significant effect on maintenance of CB $CD34^+$ cells was observed upon LPS addition to cultures supplemented with SCF, TPO, and Flt3L cytokines only (-LPS 13.37±4.36%, +LPS 17.03±0.91%) (Fig. 2 B). Importantly, as measured by CFSE dilution, TLR4-stimulated BMSCs and also TERT-immortalized MSCs retained up to 8-fold more $CD34^+$ cells in divisions 0-3 as compared to co-cultures without LPS, whereas 0-3 fold dividing $CD34^+$ cells were hardly detectable in cultures supplemented with cytokines only, irrespective of LPS addition (Fig. 2 C and Fig. S3 B).

To assay myeloid differentiation potential, remaining cells from primary cultures were subjected to cytokine supplemented CFU assays for full myeloid read out. Consistent with higher $CD34^+$ cell content, hematopoietic cells recovered from TLR4-stimulated BMSC co-cultures produced about 2.5-fold more myeloid colonies as compared to cells from un-stimulated BMSC co-cultures, and mixed myeloid and erythroid colonies could only be detected from cells derived from TLR4-stimulated BMSC co-cultures (Fig. 2 D). CFU-activity from cells that were pre-cultured in cytokines only was overall lower compared to CFU-activity from co-cultures, no mixed myeloid and erythroid colonies could be detected, and no relevant difference with respect to LPS addition was observed (Fig. 2 D). To test also lymphoid cell differentiation capacity, BMSC co-cultured CB cells were transplanted into irradiated newborn $Rag2^{-/-}\gamma_c^{-/-}$ mice[30]. Low levels of human B cell and T cell engraftment was detected only in animals receiving hematopoietic cells from TLR4-stimulated BMSC co-cultures (Fig. 2 E, F and Table S1).

Together, these results demonstrate that primary human BMSCs and TERT-immortalized MSCs upon TLR4-agonist stimulation produced soluble or membrane-bound factors that increased maintenance of immature human hematopoietic cells with myeloid and lymphoid developmental potential.

TLR4 stimulation induces bone marrow *G-csf* mRNA transcripts, and TLR4 stimulation induced G-CSF serum levels depend mostly on non-hematopoietic cells *in vivo.*

TLR4 is expressed on both hematopoietic cells as well as some non-hematopoietic cells, and both hematopoietic cells and non-hematopoietic tissues are capable to produce myeloid cytokines[3-5]. Given this and the above described data that human BMSCs and TERT-immortalized MSCs upon LPS stimulation increase production of myeloid cyokines, support progenitor cells and enhance myeloid differentiation *in vitro*, we wanted to determine the relevance of these findings *in vivo*. To this end, we focused on G-CSF as key granulocyte-supporting cytokine with clinical relevance which is expressed at very low levels in steady-state and increases in bacterial induced inflammation or sepsis[6-9].

G-csf mRNA was detectable in total tissue extracts at low quantities in steady-state spleen and lung as described[31], but not in bone marrow. At 6 hours after intraperitoneal (i.p.) LPS injection, *G-csf* mRNA expression was highly up-regulated in bone marrow, the site of granulopoiesis, as well as in lung and spleen, the sites of granulocyte migration and activation, (Fig. 3 A). To determine hematopoietic cell versus non-hematopoietic cell G-CSF production, we generated chimeric mice with TLR4$^{-/-}$ hematopoiesis in a wild-type (WT) background (hematopoietic-TLR4$^{-/-}$) and wild-type hematopoiesis in a TLR4$^{-/-}$ non-hematopoietic background (non-hematopoietic-TLR4$^{-/-}$). No differences in engraftment efficiency, hemato-lymphoid organ cellularity and lineage reconstitution in either wild-type to TLR4$^{-/-}$ or TLR4$^{-/-}$ to wild-type recipient mice were observed (data not shown). Furthermore, at eight weeks after chimera generation, residual host CD45^{+} chimerism was below 5% (Fig. S4 B and data not shown). Remaining host cells consisted mostly of CD3^{+} T cells, and very few CD11c^{+} cells and F4/80^{+} cells, i.e. likely antigen presenting cells and macrophages. The low residual host F4/80^{+} cells were not obviously changed by adding clodronate, a macrophage depleting agent, into the pre-transplant conditioning regimen (Fig. S4 C).

LPS injection into WT and TLR4$^{-/-}$ control mice as well as into hematopoietic chimeric mice (Fig. S4 A) at 8 weeks after chimera generation led to bone marrow *G-csf* mRNA induction in WT, hematopoietic-TLR4$^{-/-}$, and to a much lesser extent in non-hematopoietic-TLR4$^{-/-}$ mice, while no transcripts were detectable in TLR4$^{-/-}$ control mice (Fig. 3 B). In line with the induction of *G-csf* mRNA, a significant,

approximately ten-fold increase in plasma G-CSF levels was observed at 72 hours after LPS injection in WT and hematopoietic-TLR4$^{-/-}$ mice, while no increase was detectable in non-hematopoietic -TLR4$^{-/-}$ and TLR4$^{-/-}$ mice, respectively (Fig. 3 C).

We conclude that TLR4 stimulation induces granulopoiesis supporting *G-csf* mRNA transcripts in gamma irradiation-resistant, non-hematopoietic cells in bone marrow, i.e. at the primary site of granulopoiesis. Furthermore, TLR4 stimulation induced G-CSF serum levels depend mostly on non-hematopoietic cells *in vivo***.**

Non-hematopoietic cells are sufficient and the main contributors to enhance bone marrow myeloid cell production upon TLR4 stimulation *in vivo.*

To evaluate *in vivo* hematopoietic responses upon TLR4 stimulation in WT, TLR4$^{-/-}$, and chimeric mice, LPS was injected i.p. twice in a 48 hour interval, and mice were analyzed 24 hours later (Fig. S4 A). Absolute bone marrow cell numbers decreased and spleen cell numbers increased in WT and hematopoietic-TLR4$^{-/-}$ mice, while no relevant total cell number changes were observed in TLR4$^{-/-}$ and non-hematopoietic-TLR4$^{-/-}$ mice (Fig. 4 A, B). As myeloid cell (granulocyte/monocyte/macrophage) maturation in the bone marrow can be determined by expression levels of Gr-1 and CD11b, with immature promyelocytes and myelocytes being Gr-1^{low}CD11b$^{low/+}$, and mature cells being Gr-1^{high}CD11b$^{low/+}$ [32, 33], we determined frequency changes of these cellular fractions upon LPS injection. In WT and hematopoietic-TLR4$^{-/-}$ mice, bone marrow Gr-1^{high}CD11b$^{low/+}$ mature cells decreased, while relative frequencies of immature Gr-1^{low}CD11b$^{low/+}$ cells increased up to 2.5 fold (Fig. 4 C - E). The loss of mature Gr-1^{high}CD11b$^{low/+}$ cells from bone marrow of WT and hematopoietic-TLR4$^{-/-}$ mice was accompanied by an increase of these cell numbers in spleen (Fig. 4 B and data not shown). In contrast, no change of Gr-1^{high}CD11b$^{low/+}$ mature cells was observed in TLR4$^{-/-}$ and non-hematopoietic-TLR4$^{-/-}$ mice, and no change of immature Gr-1^{low}CD11b$^{low/+}$ cells was measured in TLR4$^{-/-}$ mice, while a small, about 1,25 fold, but still significant increase of immature Gr-1^{low}CD11b$^{low/+}$ cells was detectable in non-hematopoietic-TLR4$^{-/-}$ mice (Fig. 4 C - E). Thus, while hematopoietic-expressed TLR4 might add to TLR4-agonist mediated bone marrow granulopoiesis, major effects are mediated by non-hematopoietic cells.

To determine if responses observed in promyelocytes and myelocytes would similarly occur in very early myeloid (Gr-1 negative) bone marrow cells, we analyzed the respective population patterns. It was demonstrated previously that *in vivo* LPS or

Escherichia coli challenge leads to rapid Sca-1 up-regulation on c-Kit^{+} hematopoietic progenitors in bone marrow, associated with an increase in CFU-granulocyte/macrophage activity[34, 35]. Thus, Sca-1 in this case does not delineate a hematopoietic stem cell population but can be used as an indicator for inflammation-induced changes in c-Kit^{+} progenitor patterns. Strongest increase of Sca-1 expression, associated with an increase of the Fcγ receptor-II/III (FcγR) expression was observed in WT mice, with a gradual decreased response in hematopoietic-TLR4$^{-/-}$ to non-hematopoietic-TLR4$^{-/-}$ mice, while TLR4$^{-/-}$ mice showed no relevant expression alterations (Fig. 5 A). This pattern changes were associated with a relative increase in combined short-term HSCs (ST-HSCs), common myeloid progenitors (CMPs), and granulocyte-macrophage progenitors (GMPs) in WT, a slight increase or no increase in hematopoietic-TLR4$^{-/-}$ mice, and no increase in non-hematopoietic-TLR4$^{-/-}$ and TLR4$^{-/-}$ mice. Thus, these phenotypic changes in earliest myelopoiesis are in line with findings in the downstream progeny (Fig. 4 D, E).

Given these data and previous reports demonstrating increased bone marrow myeloid CFU activity during bacterial infections in mice[9], we tested if LPS injection was also associated with a relative increase of cells reading out in myeloid CFU assays. Indeed CFU activity, and therefore progenitor cell frequency was increased in WT and hematopoietic-TLR4$^{-/-}$ mice, while no relevant change was observed in non-hematopoietic-TLR4$^{-/-}$ and TLR4$^{-/-}$ mice (Fig. 5 B). At the same time, similar as in human sepsis[7, 8], c-Kit^{+} hematopoietic precursor cells were mobilized into peripheral blood of WT and hematopoietic-TLR4$^{-/-}$ mice (being significant with the numbers of experiments performed only in WT mice), while no relevant change was observed in TLR4$^{-/-}$ mice, and only small, if any increase occurred in non-hematopoietic-TLR4$^{-/-}$ mice (Fig. 5 C).

Together this data demonstrates that LPS induced *in vivo* signaling via non hematopoietic cell expressed TLR4 is both sufficient and the main mechanism to both release mature myeloid cells from and to induce enhanced myeloid cell production in the bone marrow.

Combined TLR4 and cytokine stimulation in absence of stroma enhances granulocytic read-out from mouse but not human hematopoietic progenitor cells cells *in vitro.*

It was previously demonstrated that mouse HSC and GMP express TLR4, that LPS can force these populations into cell cycle, and can drive cytokine-independent monocyte/macrophage differentiation in liquid cell culture [10]. Analysis of human $CD34^+$ CB cells revealed *Tlr4* and *MD2* mRNA expression, similar to human peripheral blood DCs (Fig. S2 A, B).

Given our *in vitro* findings with human BMSCs and *in vivo* findings in chimeric mice on the relevance of non-hematopoietic cells in myeloid differentiation responses, we next determined direct effects of LPS on TLR4 expressing hematopoietic progenitor cells in cytokine supplemented myeloid colony forming assays using both human $CD34^+$ cord blood and mouse $lin^-c\text{-}Kit^+$ bone marrow hematopoietic progenitor cells. TLR4 stimulation in the absence of stroma did not grossly influence frequency of colony formation, however, mouse myeloid colony read out was biased towards CFU-G with reduced alternative myeloid colonies while human CFUs were not obviously altered (Fig. 6 A, B). Although it remains unclear from this assay if TLR4 agonist stimulation instructed mouse CFU-G read out at cost of other lineages or if different progenitors were recruited, we conclude that in mice, direct stimulation of TLR4 on progenitors in presence of myelopoiesis supporting cytokines can bias colony read out towards granulocytic differentiation but does not alter total CFU activity. In contrast, no such effect is seen in same conditions with human cells, suggesting different evolvement of these mechanisms during evolution of mouse and human species. The results with mouse cells are in line with hematopoietic progenitor expressed TLR4 being involved in fine-tuning myeloid responses[10], while not being sufficient for sustaining an enhanced response in absence of non-hematopoietic expressed TLRs.

DISCUSSION

A first key finding of the present study is that human BMSCs express functional TLR4 and MD-2 proteins and respond to LPS stimulation with production of factors that support maintenance of human hematopoietic progenitor cells with myeloid and lymphoid differentiation capacity as well as the formation of mature myeloid innate immune effector cells. While maintenance of early progenitors is likely achieved in

part by direct cell contact via e.g. induced membrane bound cytokines as stem cell factor and Flt3-L, highly induced and secreted soluble factors as G-CSF, M-CSF, and GM-CSF support myeloid cell differentiation.

A second key finding is that LPS mediated TLR4-signaling induces a high increase of *G-csf* mRNA transcripts in non-hematopoietic cells in bone marrow, that subsequently increased serum G-CSF protein levels depend on production by non-hematopoietic cells, and that non-hematopoietic cell TLR4-signaling is both sufficient and the main mechanism to both release mature myeloid cells from and to induce enhanced myeloid cell production in bone marrow *in vivo*.

Tlr mRNA expression-profiles were recently reported in two studies on human and one study on mouse MSCs that, with the exception of strong *Tlr2* and *Tlr8* expression in mouse MSCs, correlate closely to our data[36-38]. The studies demonstrated human MSCs migration *in vitro* towards TLR3-agonists[38], and reduced *in vitro* T cell inhibitory activity of human MSCs upon TLR3 and TLR4 ligation, which was in part dependent on down-regulation of MSC-expressed Notch ligand Jagged-1[36]. *In vitro* TLR2 ligation on mouse MSCs induced their proliferation and inhibited differentiation into mesodermal derivates, but did not reduce their ability to inhibit *in vitro* T cell proliferation[37]. Thus, TLR ligation on MSCs might influence their migratory, proliferation, differentiation, and adaptive immune system modulatory capacities *in vivo*. However, as BMSCs are mostly sessile cells with little turn-over, and the bone marrow is infrequently a site of initiation of adaptive immune responses, our data bring to mind that a main *in vivo* function of BMSCs likely lies in the primary support and regulation of hematopoiesis.

The process of induced granulo-monocytic responses, also called “emergency granulopoiesis”, i.e. the mobilization of granulo-monocytic cells from bone marrow to blood followed by increased bone marrow production of these cells upon inflammatory signals, has been the focus of several recent studies. Steady-state granulopoiesis is supported by IL-3, IL-6, G-CSF, and GM-CSF[2-5], with G- and GM-CSF serving as primary regulators, as respective knockout mice show defects in both production and function[39-41]. And these cytokines are most likely involved in inflammation induced granulopoiesis as they are increased upon *in vivo* microbial infections in both mice and humans and lead to increase granulocyte progenitor proliferation[7-9, 42, 43]. However, alternative and complementary pathways for discrete inflammation induced enhancement of granulopoiesis exist, as mice with single or

combined deficiencies for G-CSF, G-CSF and IL-6, or G-CSF and GM-CSF are still able to mount reactive neutrophilia responses[40, 41, 43, 44]. One such pathway in alum or urea induced granulopoiesis might act via the Nalp3 inflammasome, inducing IL-1 production that in turn leads to induction of multiple growth factors and inflammatory mediators[45, 46]. Interestingly, it recently was demonstrated that alum induced neutrophilia is regulated by TNFα induced down-regulation of SCF and SDF-1 (CXCL12) that leads to lymphocyte liberation of bone marrow niche space, giving a competitive advantage to granulopoiesis to assess IL-1 mediated cytokine increase[33, 47] which is selectively inhibited in non-hematopoietic IL-1R Type I deficient mice[48]. Indeed, *in vitro* IL-1β stimulation of human BMSCs and human TERT-immortalized MSCs lead to high level induction of IL-6 and G-CSF, comparable to the levels reached in supernatants of LPS stimulated cells, while stimulation with TNFα induced only about one log lower increases of these cytokines (data not shown). However, as selective stimulation of hematopoietic-expressed TLR4 was not leading to a robust myelopoietic response *in vivo*, we conclude that hematopoietic, i.e. monocyte and macrophage produced IL-1 is insufficient for induction of reactive myelopoiesis supporting factors from non-hematopoietic cells upon endotoxin-exposure. Thus, the relative and synergistic contribution of IL-1R versus TLR4 ligation on non-hematopoietic cells during inflammatory myeloid responses will need to be addressed in future studies.

Another pathway for inflammation-induced myeloid responses might act through a direct, hematopoietic cell intrinsic pathway. Is has been demonstrated that mouse HSCs as well as myeloid and lymphoid restricted progenitors express a diverse repertoire of TLRs[10]. TLR2 and TLR4 stimulation leads to dendritic cell differentiation at cost of B cell differentiation from lymphoid-biased progenitors and to monocyte and macrophage differentiation from myeloid progenitors, bypassing their usual need for M-CSF and GM-CSF for respective differentiation [10]. Furthermore, *in vivo* herpes infection biased lymphoid progenitors towards dendritic cell differentiation, likely via a direct, lymphoid progenitor expressed TLR9-dependent pathway[49]. Also, it has been demonstrated that HSCs enter circulation and traffic through the lymphatic system[50, 51]. Under defined experimental conditions, tissue-trafficking HSCs were able to differentiate to innate immune cells via TLR activation at extra-medullary sites, a mechanism that could be involved in local control of infections[50, 52]. Our experiments extend this data by demonstrating that combined cytokine and TLR4 stimulation can

enhance *in vitro* granulocytic read out, at least from mouse progenitor cells. The mechanism remains to be determined, but we speculate that it might be mediated through TLR4 signaling induced up-regulation of respective cytokine receptors on progenitor cells. Thus, while direct TLR4 stimulation pathways might play an important role in fine-tuning the myeloid differentiation responses in bone marrow and at sites of infection, the here demonstrated *in vivo* data unambiguously shows that hematopoietic stem and progenitor cell expressed TLR4 alone is insufficient to induce a major granulo-monocytic emergency response at primary hematopoietic sites.

A long-standing question in hematopoiesis is whether growth factor induced signaling is sufficient to induce cell fate decisions in early hematopoietic progenitors, or if progenitors are produced at equal rates, and differentiation or death is regulated by availability or lack of cytokines, respectively[53]. Although several studies suggested instructive lineage signalling using cytokine receptor over-expression[54, 55], a clear case for lineage instruction was only recently made by single cell tracking of myeloid progenitors exposed to GM-CSF (leading to granulocyte production) versus M-CSF (leading to macrophage production)[56]. The here demonstrated at least two-fold increase in myeloid colony formation from an identical starting number of human $CD34^+$ CB progenitors upon use of myeloid differentiation cytokine rich supernatant from LPS-stimulated BMSC could either be due to recruitment of otherwise dying progenitors, or to the redirection of otherwise pre-committed progenitors to the granulo-monocytic lineage. As the total CFU read-out of plated cells did not exceed 65%, these questions will need to be addressed with pre-selection of lineage-primed populations[28, 55, 57, 58].

Based on the presented data, we here propose a graded model of the innate myeloid cell immune response which evolved to protect from severe infections: Local infection leads to vasculature egress of myeloid cells, a process that involves e.g. endothelium-expressed TLR4 in the lung, that upon LPS stimulation up-regulate P selectin expression[13, 14]. At the same time, myelopoiesis active cytokines produced at infectious sites as well as locally present TLR-agonists support at site hematopoietic cell differentiation and maturation[10, 50]. In more extended infections, with sepsis being the maximal variant, bacterial products reach the primary site of myeloid cell production, i.e. the bone marrow, and stimulate TLR-expressing BMSCs that, via secreted cytokines and direct interaction with hematopoietic progenitor cells,

enhance myeloid differentiation. At the same time, BMSCs produce factors that maintain progenitor cells, thereby preventing exhaustion of this cell pool during emergency responses (Fig. 7). Similar to the here studied gram-negative bacteria expressed TLR4-agonist LPS, other systemic infection and inflammation induced TLR4-agonists such as heat shock proteins and fibrinogen could induce equivalent effects[10, 12]. Furthermore, relatively high mRNA expression of *Tlr1, Tlr5,* and *Tlr6* in BMSCs suggest that other microbial pathogen compounds might elicit similar responses.

While this model is strongly supported by the here presented *in vitro* and *in vivo* analysis using both, human and mouse experimental systems, the exact *in vivo* non-hematopoietic cell correlates and their specific location in the bone marrow microenvironment will need to be clarified. Recent progress in prospective isolation and transplantation of mouse multipotent mesenchymal stem cells might help to address these issues[59]. Upon identification of appropriate targets, selective delivery of TLR agonists to bone marrow non-hematopoietic cells with the intention to broadly enhance myeloid cell regeneration could then be an intriguing future therapeutic possibility, supplementing current application of single cytokines.

MATERIAL AND METHODS

Cell samples. Cord blood from healthy full-term newborns was obtained upon written informed parental consent. Bone marrow samples were obtained upon written informed consent from patients undergoing orthopedic joint replacement surgery. The use of cord blood and bone marrow was approved by the Cantonal Ethics Board of Ticino, Switzerland. Peripheral blood mononuclear cells were obtained from healthy voluntary blood donors.

Isolation and sorting of cord blood $CD34^+$ cells. Mononuclear cells from cord blood were isolated by density gradient centrifugation (Ficoll-Paque; ICN Biomedicals, Frankfurt am Main, Germany). $CD34^+$ cells were immuno-magnetically enriched according to the manufacturer's instructions ($CD34^+$ selection kit, Miltenyi Biotec, Bergisch-Gladbach, Germany), and subsequently sorted as $CD34^+$ propidium iodide$^-$ (PI^-) cells using a FACSAria flow cytometer (Becton Dickinson, Franklin Lakes, USA).

Isolation and immunophenotypic characterization of BMSCs. Human BMSCs were isolated by plastic adherence from bone marrow. After Ficoll gradient purification, cells were plated in FCS pre-coated T75 flasks in Iscove's Modified Dulbecco's Medium (IMDM) with 15% FCS (HyClone defined, Logan, USA), penicillin (50 U/ml; GIBCO, Carlsbad, USA), streptomycin (0.05 mg/ml; GIBCO, Carlsbad, USA) and dexamethasone (10^{-8} M; Sigma-Aldrich, St. Louis, USA)[60]. After overnight incubation, non-adherent cells were washed off from flasks and adherent cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 30% FCS (HyClone defined), penicillin (50 U/ml; GIBCO), and streptomycin (0.05 mg/ml; GIBCO) for 1-3 weeks (medium change every 3 days) to near confluence. Using trypsin (Trypsin–EDTA 0.05%, 5 min incubation) cells were removed and re-plated at a density of 50 cells/cm^2 in DMEM containing 10% FCS, penicillin (50 U/ml;GIBCO) and streptomycin (0.05 mg/ml; GIBCO) into new flasks and grown to conflucence. BMSC surface phenotype was assessed at passage 1 using antibodies against the following antigens: CD45 (clone HI30), CD105 (SN6), CD73 (AD2), CD34 (581), CD90 (5E10), CD19 (HIB19), CD14 (RM052), CD117 (104D2), HLA-DR (Tue36), CXCR4 (12G5). Antibodies were purchased from BD Biosciences (San Jose, USA) or eBiosciences (San Diego, USA). Dead cells were excluded by propidium iodide staining and isotype-matched control antibodies were used to determine background staining.

Differentiation of BMSCs. For all differentiation assays, BMSCs were grown to confluence in 60 mm cell culture dishes**.** For adipogenic differentiation, BMSCs at confluence were cultured with adipogenic induction medium consisting of DMEM containing 4.5g/L glucose, 10% FCS (HyClone defined), 1μM dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-Isobutyl-1-methyl-xanthin, 0.01 mg/ml insulin (all reagents from Sigma-Aldrich), penicillin (50 U/ml; GIBCO) and streptomycin (0.05 mg/ml; GIBCO) for 21 days with complete medium exchange every 3 days. For staining of lipid vacuoles, BMSCs were washed with PBS twice and incubated in freshly filtered Oil Red O solution (Sigma-Aldrich) for 30 minutes. For osteogenic differentiation, BMSCs were cultured in osteogenic induction medium consisting of DMEM supplemented with 10% FCS, 0.1 μM dexamethasone, 0.05 μM L-Ascorbic-acid 2 phosphate, 10 mM glycerol 2-phosphate (all reagents from Sigma-Aldrich), penicillin (50 U/ml; GIBCO) and streptomycin (0.05 mg/ml; GIBCO) for 30 days with a complete medium exchange every 3 days. Calcium deposits were detected with von Kossa staining: cell layers were fixed with 10% formalin (Sigma-Aldrich) for 1 h,

incubated with 2% silver nitrate solution (w/v, Sigma-Aldrich) for 10 min in the dark, washed thoroughly with deionized water, and then exposed to bright light for 15 min.

RNA isolation and RT-PCR analysis. For the isolation of RNA, tissue or cells were lysed in TRIzol reagent and RNA was purified according to the manufacturer`s instructions (Invitrogen, Carlsbad, USA). All RNA samples were subjected to DNAse I (Invitrogen) treatment. RNA from BMSCs was used from passage 1 to passage 3 cells. For the isolation of RNA from human dendritic cells (DC) and natural type I interferon producing cells (IPC), buffy coats from healthy donors were immunomagnetically pre-enriched for BDCA-1^+, and BDCA-4^+ cells with fluorescein isothiocyanate-conjugated and phycoerythrin-conjugated antibodies to BDCA-1 (AD5-8E7) and BDCA-4 (AD5-17F6) and anti-fluorescein isothiocyanate-conjugated and anti-phycoerythrin microbeads (all Miltenyi Biotech). Immunomagnetically enriched cells were subsequently sorted as BDCA-1^+, $CD14^-$, $CD19^-$ (DCs), and BDCA-4^+, $CD14^-$, $CD19^-$ (IPCs) cells. For the isolation of RNA from mouse tissue, mice were sacrificed 6 hours after PBS or LPS (Ultrapure, InvivoGen, San Diego, USA) injection by CO_2 inhalation. Tissue samples from spleen and lung, were immediately homogenized with ultra-turrax T8 followed by TRIzol extraction. BM samples (femur and tibia pooled) were first crushed in PBS using mortar and pestle and further homogenized with ultra-turrax T8 in TRIzol. cDNA was synthesized using random hexamers or Oligo$(dT)_{12\text{-}18}$ Primer and Superscript III reverse transcriptase according to the manufacturer`s instructions (Invitrogen, Carlsbad, USA). For BMSCs 50ng cDNA each was analyzed using the following primer sequences and annealing temperatures: G-CSF-F ACTCTGGACAGTGCAGGAAG, G-CSF-R AGGTGGC-GTAGAACGCGGTA; M-CSF-F ACAGTCAGATGGAGACCTCG, M-CSF-R TCTTGACCTTCTCCAGCAAC; FLT3L-F ACAACCTATCTCCTCCTGCTG, Flt3L-R GGCACATTTGGTGACAAAGTG (all t_m=55°C). GM-CSF-F CTGCTGCT-GAGATGAATGAA; GM-CSF-R GCACAGGAAGTTTCCGGGGT (t_m=50°C). TPO-F GACCAAGGCACAGGACATTC, TPO-R GCAGACCAGGAATCTTGGCT; IL-6-F GTAGCCGCCCCACACAGACAGCC, IL-6-R GCCATCTTTGGAAGGTTCAGG (all t_m=60°C). GAPDH-F GCCAAGGTCATCCATGACAACTTTGG; GAPDH-R GCCTGCTTCACCACCTTCTTGATGTC (t_m=50, 55, 60°C). For positive control cDNA from total bone marrow cells or teratocarcinoma NT-2 cell line [61] (gift from Hans-Joerg Buehring, Tuebingen) was amplified. cDNA was amplified using REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich) and consisted of an initial denaturation

step at 94°C for 2 minutes, followed by 37 cycles at 94°C for 1 minute, annealing for 1 minute, and extension at 72°C for 1 minute in each cycle. PCR products were diluted as indicated in Figure 1B and electrophoresed on an ethidium bromide-stained 1.5% agarose gel.

Real-time PCR for *Tlr* mRNA expression in human BMSCs, human TERT-immortalized MSCs, PB IPCs, PB DCs and CB $CD34^+$ cells and *G-csf* mRNA expression in mouse tissues was performed using a sequence detector (ABI PRISM 7700; PerkinElmer, Waltham, USA) and TaqMan target mixes (Assay-on-Demand Gene expression reagents; Applied Biosystems, Carlsbad, USA). Expression levels are given as arbitrary units relative to endogenous 18S RNA.

ELISA. Cytokine and chemokine production of control and LPS stimulated BMSCs as well as human TERT-immortalized MSCs was assayed by ELISA. BMSCs at passage 1 were grown to confluence in a T75 flask and kept for 48 hrs in 7ml of IMDM (GIBCO) supplemented with 20% FCS, 2mM L-glutamine, 50 nM 2-mercaptoethanol (all reagents from Sigma-Aldrich), antibiotics (GIBCO) and with or without LPS (10μg/ml, Invivogen) and/or CpG (1 μM, Microsynth) stimulation. Supernatants were harvested, cleared by centrifugation and passed through a 0.45μm filter. Culture supernatants from LPS, CpG and un-stimulated BMSCs were analyzed for G-CSF, GM-CSF, M-CSF, IL-6, IL-11, SCF, SDF-1 and IL-7 by ELISA according to the manufacturer's instructions (all R&D systems, Minneapolis, USA). Measurement of mouse G-CSF was done in serum of LPS versus PBS treated C57BL/Ka-Thy1.1 (CD45.1), C57BL/6 TLR-$4^{-/-}$ (CD45.2), hematopoietic-$TLR4^{-/-}$ and non-hematopoietic-$TLR4^{-/-}$ mice according to the manufacturers instruction (R&D systems).

Methylcellulose-Assays. Methylcellulose assays were performed as described[28, 55]. Specifically, to asses CFU supporting capacity of BMSC and human TERT-immortalized MSC supernatants, supernatants either from LPS stimulated or non-stimulated BMSCs and human TERT-immortalized MSCs were prepared as specified for ELISA and mixed with methylcellulose (Methocult H4100, 2.6%, StemCell Technologies, Vancouver, Canada) to yield a final concentration of 0.9% methylcellulose. Cytokines were added in the following concentrations: huSCF (10 ng/ml), huFlt3L (10 ng/ml) and huTPO (50 ng/ml) (all samples); LPS (10 μg; InvivoGen) (non stimulated BMSC supernatant 1); huM-CSF (10 ng/ml), huG-CSF

(900 pg/ml), and huGM-CSF (900 pg/ml) (non-stimulated BMSC supernatant 2) (all cytokines from R&D Systems).

To assess overall colony forming capacity of cord blood $CD34^+$ cells, IMDM was supplemented with 20% FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (all reagents from GIBCO) mixed with methylcellulose and the following cytokines added: huSCF (10 ng/ml), huFlt3L (10 ng/ml), huTPO (50 ng/ml), huIL-3 (20 ng/ml), huIL-6 (10 ng/ml), huIL-11 (10 ng/ml), huGM-CSF (50 ng/ml), and human erythropoietin (huEPO, 4 U/ml) (all R&D systems). For negative controls, methylcellulose was mixed with IMDM (20% FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol), supplemented with huSCF (10 ng/ml), huFlt3L (10 ng/ml), huTPO (50 ng/ml) and LPS (10 μg/mL).

To asses CFU capacity of cultured $CD34^+$ cells, human hematopoietic cells recovered from co-culture on BMSCs or from cytokine supported culture were added at a density of 2000 cells per mL to a methylcellulose/IMDM (20% FCS 2 mM L-glutamine, 50 μM 2-mercaptoethanol) premix including complete human cytokine supplementation as described above. For evaluation of CFU activity from $Lin^-c\text{-}Kit^+$ WT mouse bone marrow and from bone marrow of WT, $TLR4^{-/-}$ and chimeric mice after PBS or LPS stimulation in vivo cells were plated in methylcellulose (Methocult M3231, StemCell Technologies) mixed with IMDM (30% FCS. 2 mM L-glutamine, 50 μM 2-mercaptoethanol) with the following cytokines added: mIL-3 (10 ng/ml), hIL-6 (10 ng/ml), mSCF (10 ng/ml), mGM-CSF (10 ng/ml), mTPO (50 ng/ml) and huEPO (2 U/ml) (all R&D systems). In case of bone marrow from PBS or LPS treated mice, total nucleated BM cells from two pooled hind legs were plated at a density of $3x10^4$ cells per well.

Cell culture. $CD34^+$ cells ($5x10^3$/200μl) were cultured for 12 days in 96 well plates in IMDM supplemented with penicillin (50 U/ml; GIBCO) and streptomycin (0.05 mg/ml; GIBCO), 50 nM β-mercaptoethanol and 10% FCS, and SCF (10 ng/ml), Flt3L (10 ng/ml), TPO (50 ng/ml), or co-cultured on confluent BMSCs (passage 1) or human TERT-immortalized MSCs and with or without LPS (10 μg). To determine divisions of $CD34^+$ cells in culture, cells were labeled for 15 min with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) at 37°C before plating. Cells from cytokine supplemented cultures and co-cultured cells were used for FACS-analysis, secondary CFU-assays and transplantation. After 12 days of culture, co-

cultured, non adherent cells (NA-1) were removed, followed by trypsin release of adherent cell layers. Trypsinized cells were washed with IMDM supplemented with penicillin (50 U/ml;GIBCO), streptomycin (0.05 mg/ml; GIBCO), 50 nM β-mercaptoethanol, 10% FCS and replated in T25 flasks for 30 min at 37°C. After incubation nonadherent cells (NA-2) were removed and mixed with non adherent cells (NA-1) from the first harvesting step. For transplantation experiments hematopoietic cells were pooled from several 96 wells.

Mice. C57BL/Ka-Thy1.1 (CD45.1, WT), C57BL/6 TLR4$^{-/-}$ [62] (CD45.2), and BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ [30], mice were maintained at the Institute for Research in Biomedicine animal facility and treated in accordance with guidelines of the Swiss Federal Veterinary Office. Experiments were approved by the Dipartimento della Sanità e Socialità, Ticino, Switzerland.

Newborn transplantation assay. Newborn Rag2$^{-/-}\gamma_c^{-/-}$ mice were irradiated in a 4 hour interval with 2x2 Gy from a Cesium 137 source (Biobeam 8000, STS GmbH, Braunschweig, Germany) at 3.75 Gy/min as previously described [30]. At 2 hours post irradiation, mice were transplanted with hematopoietic cells recovered from co-cultures in 25µl PBS into the liver (i.h.) using a 30-gauge needle (Hamilton Bonaduz AG, Bonaduz, Switzerland). Mice were weaned at 3 weeks and sacrificed at 4 weeks of age. Mice were weaned at 3 weeks and sacrificed at 4 weeks of age.

Generation chimeric mice. Bone marrow was obtained from femurs of respective female C57BL/Ka-Thy1.1 (CD45.1) or C57BL/6 TLR-4$^{-/-}$ (CD45.2) donor mice. Bone marrow samples were immunomagnetically pre-enriched for c-Kit^{+} cells with allophycocyanin-conjugated antibody to c-Kit (2B8; eBioscience) and anti-allophycocyanin microbeads (Miltenyi Biotech). Cells were then stained with phycoerythrin-indodicarbocyanine-conjugated antibodies to lineage antigens CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), CD19 (MB19-1), Gr-1 (RB6-8C5), Ter119 (Ter119) (all from eBioscience), and NK1.1 (PK136; Becton Dickinson). Cells were sorted as lin^{-}c-Kit^{high}. Six week old female C57BL/Ka-Thy1.1 (CD45.1) or C57BL/6 TLR4$^{-/-}$ (CD45.2) mice were lethally irradiated with 13 Gy total body irradiation and were transplanted with 3-5x10^4 lin^{-}c-Kit^{high} cells via the retro orbital venous sinus. The reconstituted mice were maintained for 8 weeks to allow for complete engraftment with donor cells which was considered being the case if

peripheral blood donor cell frequency exceeded 95% of total cells. To assay reconstitution of recipient mice peripheral blood was collected 6 weeks after transplantation. Peripheral blood was incubated twice with 5 volumes of ammonium chloride solution and subsequently washed with PBS. Cells were stained with CD45.1 fluorescein isothiocyanate-conjugated and CD45.2 allophycocyanin-conjugated antibodies to determine donor/recipient chimerism. Mice with more than 95% donor chimerism in blood at 8 weeks after transplantation were injected twice i.p. with 35 μg of LPS from E.coli (Ultrapure, InvivoGen, San Diego, USA) in a 48 hour interval and sacrificed 24 hrs after the second injection. For detection of *G-csf* mRNA, mice received a single injection of 35 μg LPS and were sacrificed 6 hours later.

Analysis of mice. To obtain peripheral blood cells and plasma, mice were bled from the retro orbital venous sinus under anesthesia. When sacrificed, single cell suspensions from organs were prepared and red blood cells were lysed. For FACS analysis of transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice, monoclonal antibodies biotinylated or conjugated against the following antigens were used: CD3 (UCHT1), CD4 (13B8.2), CD8 (B9.11) (Imunotech/Beckman Coulter, Marseille, France), CD19 (HIB19), CD34 (581), IL-3Rα/CD123 (9F5) BD Biosciences, San Diego, USA) and CD45 (HI30) (Caltag, Carlsbad, USA). For FACS analysis of WT C57BL/Ka-Thy1.1 (CD45.1), C57BL/6 $TLR4^{-/-}$ (CD45.2), hematopoietic-$TLR4^{-/-}$, and non-hematopoietic-$TLR4^{-/-}$ mice the following antibodies were used: CD3ε (145-2C11), CD19 (MB19-1), Gr-1 (RB6-8C5), CD11b (M1/70) CD45.1 (A20), CD45.2 (104) and c-Kit (ACK2) (all eBioscience). Staining for mouse stem and progenitor cells was performed using the following antibodies: CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), CD19 (MB19-1), CD11b (M1/70), Gr-1 (RB6-85C), Ter119 (Ter119) and CD127 (A7R34), c-Kit (2B8), Sca-1 (D7), CD34 (RAM34; all eBioscience) and CD16/32 (FcγRII/III) (2.4G2; Becton Dickinson).

Statistical analyses. Significance of differences was analyzed with a two-tailed Students *t*-test. A difference between experimental groups was considered as significant when the P value was <0.05. All statistical analyses were calculated with Prism software (GraphPad Software, version 4.0).

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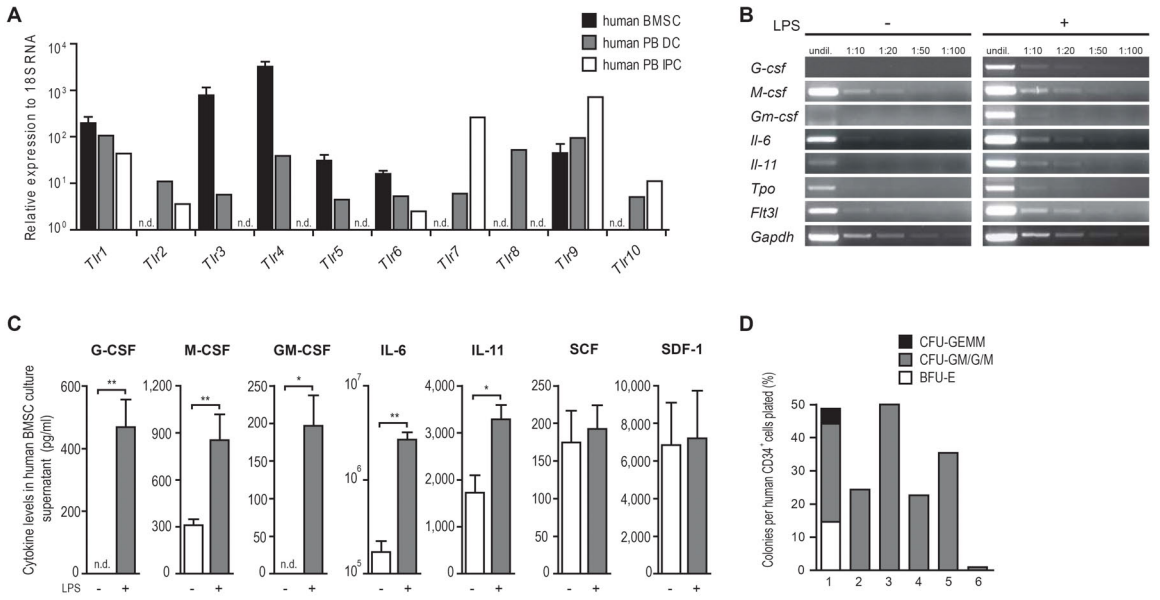

Figure 1. Human Bone Marrow Stromal Cells (BMSCs) express *Tlr* mRNA and increase production of myelopoiesis supporting cytokines upon TLR4 ligation.

(A) Bar graphs show mRNA expression of *Tlr1-10* in human BMSCs (black bars), peripheral blood dendritic cells (PB DC, grey bars) and natural interferon producing cells (PB IPC, white bars). Expression levels are given as arbitrary units relative to endogenous 18S RNA. n.d., not detectable within 35 cycles of amplification. Mean ± SEM of a representative out of three independent experiments each performed with three different bone marrow donors and one buffy coat donor are shown. **(B)** Semiquantitative RT-PCR analysis of cytokines supporting myeloid differentiation and progenitor maintenance in LPS stimulated (10 μg/ml, 24h) versus un-stimulated human BMSCs. Representative results from one independent experiment out of three each performed with different bone marrow donors are shown. **(C)** Bar graphs show cytokine and chemokine levels in supernatants taken from un-stimulated (white bars) and LPS stimulated (10 μg/ml, 48h, grey bars) BMSC cultures. Mean ± SEM of supernatants from five experiments with each different donor BMSCs are shown. n.d., not detectable with sensitivity of the assay. Statistically significant differences are indicated (*p<0.05, **p<0.01). **(D)** Bar graphs show myeloid colony–forming unit (CFU) activity of sorted human cord blood $CD34^+$ cells stimulated with SCF, Flt3L, TPO, IL-3, IL-6, IL-11, GM-CSF, and EPO (1), and with SCF, Flt3L, TPO, and supernatants from un-stimulated (2) and LPS stimulated BMSCs (3). Controls included un-stimulated BMSC supernatant with addition of SCF, Flt3L, TPO and LPS (4), un-stimulated BMSC supernatant with addition of SCF, Flt3L, TPO, and 10 ng/ml M-CSF, 900 pg/ml G-CSF, and 300 pg/ml GM-CSF (5), and cultures with only SCF, Flt3L, TPO and LPS added (6). BFU-E, burst-forming units/erythroid (white); CFU-GM/G/M, CFU-granulocyte/macrophage, -granulocyte, -macrophage (gray). CFU-GEMM, CFU-granulocyte erythrocyte monocyte macrophage (black). Five hundred human $CD34^+$ cells were plated per well. One representative out of three independent experiments with each different $CD34^+$ cells and different BMSC donors is shown.

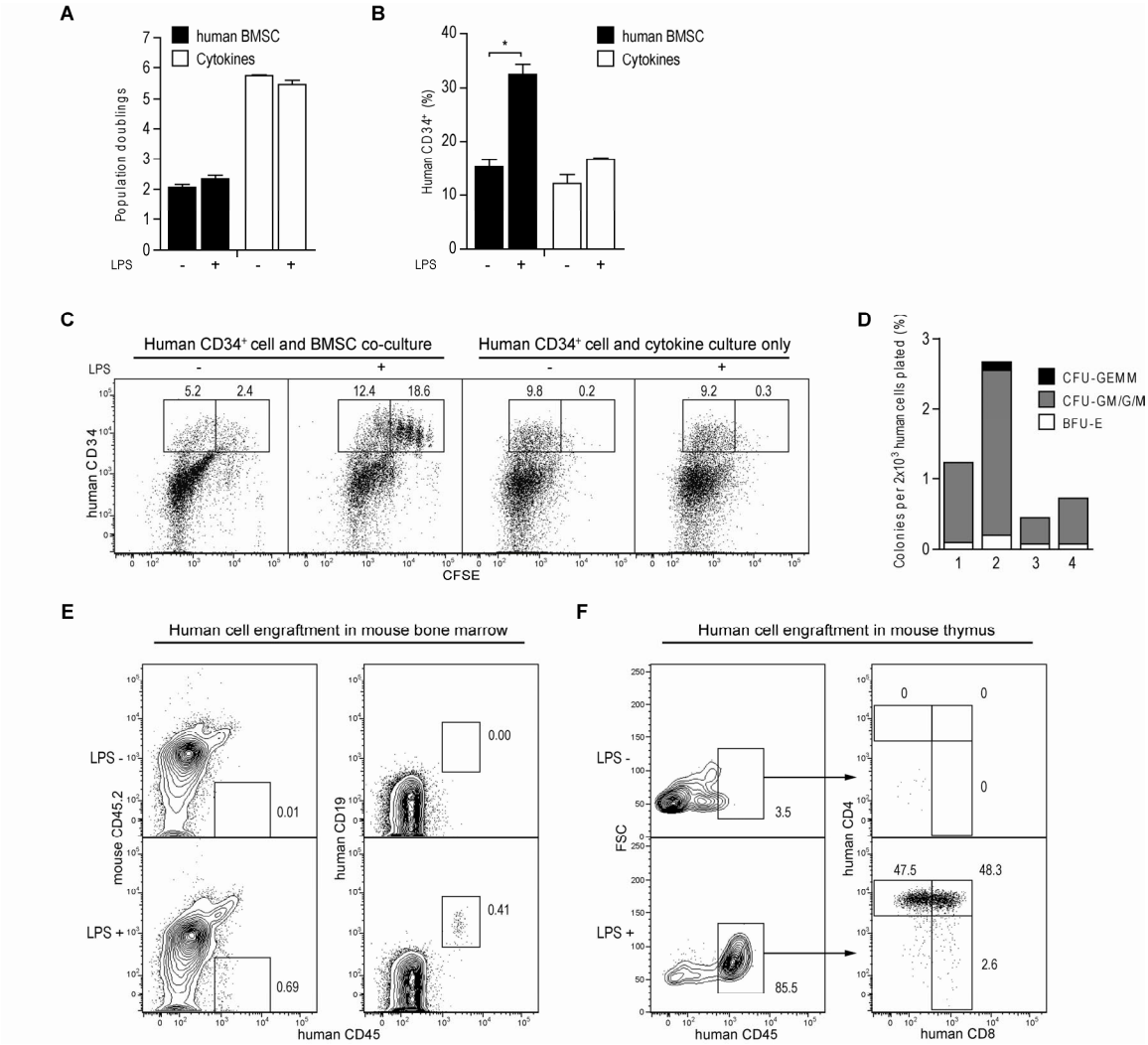
A
human BMSC
Cytokines
Population doublings
LPS
B
human BMSC
Cytokines
Human CD34+ (%)
LPS
C
Human CD34+ cell and BMSC co-culture
Human CD34+ cell and cytokine culture only
LPS
5.2
2.4
12.4
18.6
9.8
0.2
9.2
0.3
human CD34
CFSE
D
CFU-GEMM
CFU-GM/G/M
BFU-E
Colonies per 2x10³ human cells plated (%)
E
Human cell engraftment in mouse bone marrow
LPS -
LPS +
mouse CD45.2
human CD19
0.01
0.69
0.00
0.41
human CD45
F
Human cell engraftment in mouse thymus
LPS -
LPS +
FSC
human CD45
3.5
85.5
human CD4
human CD8
0
0
0
47.5
48.3
2.6

Figure 2.

Figure 2. TLR4-stimulated human BMSCs maintain human early hematopoietic progenitors with myeloid and lymphoid differentiation capacity.

(A) Population doublings of sorted PI^- human cord blood $CD34^+$ cells cultured for 12 days on human BMSCs (black bars) or in the presence of cytokines (SCF, TPO, Flt3L, white bars) without BMSCs. LPS (10 μg/ml) was added from the beginning of cultures as indicated. Population doublings were calculated according to the following equation: number of population doublings = Log (N/No) x 3.33 where: N=number of viable cells at the end of a period of growth. No=number of cells plated. Mean ± SD of three independent experiments with each different $CD34^+$ cord blood cells and different BMSC donors is shown. **(B)** Human $CD34^+$ cell percentages recovered after 12 day culture of CB $CD34^+$ cells on BMSCs (black bars) or in the presence of cytokines only (SCF, TPO, Flt3L, white bars) with or without LPS (10 μg/ml) as indicated. Mean ± SD of three independent experiments with each different $CD34^+$ cord blood cells and different BMSC donors is shown. **(C)** FACS plots show proliferation of $CD34^+$ cord blood cells by dilution of CFSE at day 12 of culture on BMSCs or in the presence of cytokines only (SCF, TPO, Flt3L) with or without addition of LPS (10 μg/ml) as indicated. Gates and numbers indicate percentage of $CD34^+$ cells that divided $\leq$3 and $\geq$4 times. Results from one representative out of three independent experiments with each different $CD34^+$ cord blood cells and different BMSC donors are shown. **(D)** CFU activity (%) of hematopoietic cells plated after 12 days of culture as described in (A). Cells ($2x10^3$ plated per well) were stimulated with SCF, Flt3L, TPO, IL-3, IL-6, IL-11, GM-CSF, and EPO. Bar graphs show colony offspring of cells cultured on BMSCs without LPS (1), with LPS (2), and cells cultured in the presence of cytokines only without LPS (3) and with LPS (4). One representative experiment out of three with each different $CD34^+$ cord blood cells and different BMSC donors is shown. **(E) and (F)** Flow cytometric analysis of the bone marrow and thymus of $Rag2^{-/-}\gamma_c^{-/-}$ mice analyzed four weeks after receiving transplants of $2\text{-}3x10^5$ human hematopoietic cells recovered after 12 days of culture on BMSCs in the presence or absence of LPS. Representative analysis out of two independent experiments with five transplanted mice per experiment is shown (see Table S1).

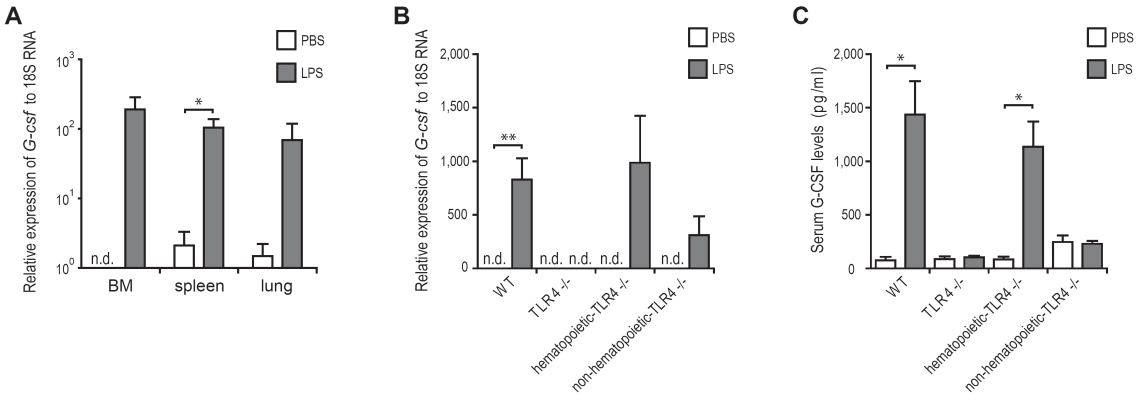
A
Relative expression of G-csf to 18S RNA
PBS
LPS
n.d.
*
BM
spleen
lung
B
Relative expression of G-csf to 18S RNA
PBS
LPS
**
n.d.
WT
TLR4 -/-
hematopoietic-TLR4 -/-
non-hematopoietic-TLR4 -/-
C
Serum G-CSF levels (pg/ml)
PBS
LPS
*
*
WT
TLR4 -/-
hematopoietic-TLR4 -/-
non-hematopoietic-TLR4 -/-

Figure 3.

Figure 3. TLR4 stimulation induces bone marrow *G-csf* mRNA transcripts and induced G-CSF protein production depends mostly on non-hematopoietic cells *in vivo.*

(A) Bar graphs show *G-csf* mRNA expression in the bone marrow (hind legs), spleen, and lung of PBS (white bars) or LPS (grey bars) treated wild type (WT) mice. Expression levels are given as arbitrary units relative to endogenous 18S RNA. Mice were i.p. injected once with 35 µg LPS and sacrificed 6 hours after the injection. n.d., not detectable within 35 cycles of amplification. Mean ± SEM of one experiment with three different mice are shown. **(B)** Bar graphs show *G-csf* mRNA expression in the bone marrow of PBS (white bars) or LPS (grey bars) treated WT, TLR4$^{-/-}$, hematopoietic-TLR4$^{-/-}$ and non-hematopoietic-TLR4$^{-/-}$ chimeric mice. Mice were treated as described in (A). Expression levels are given as arbitrary units relative to endogenous 18S RNA. Statistically significant differences are indicated (*p<0.05, **p<0.01, ***p<0.001). Mean ± SEM of three independent experiments with three mice for PBS and five mice for LPS treatment per group are shown. **(C)** Bar graphs show serum G-CSF protein levels in PBS (white bars) or LPS (grey bars) treated WT, TLR4$^{-/-}$, hematopoietic-TLR4$^{-/-}$, and non-hematopoietic-TLR4$^{-/-}$ chimeric mice which were i.p. injected twice with 35 µg LPS in an 48 hour interval and sacrificed 24 hours after the second injection. Mean ± SEM of three independent experiments with each three PBS and four LPS-treated mice per group are shown.

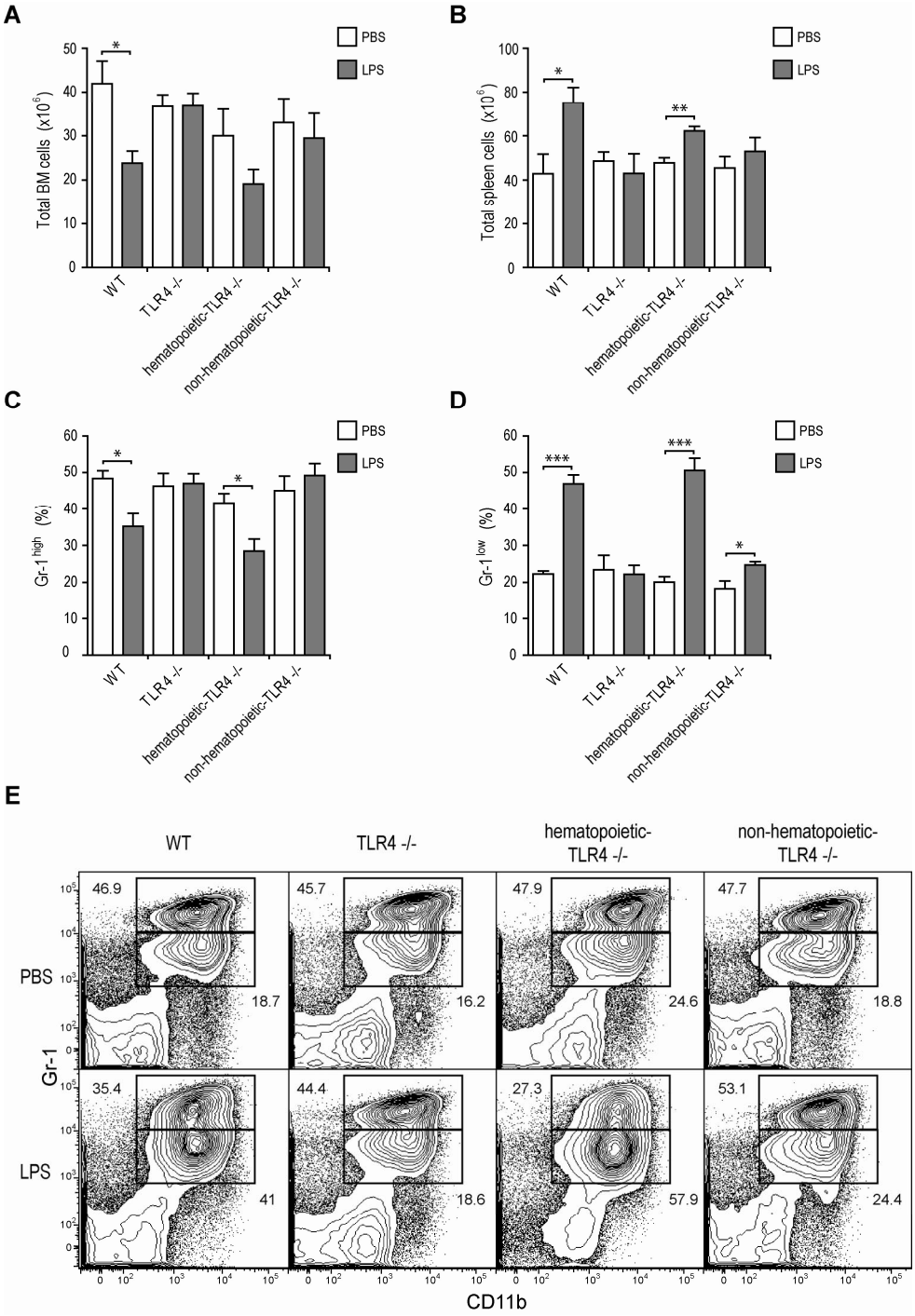
A
B
C
D
E
PBS
LPS
Total BM cells (x10^6)
Total spleen cells (x10^6)
Gr-1^high (%)
Gr-1^low (%)
WT
TLR4 -/-
hematopoietic-TLR4 -/-
non-hematopoietic-TLR4 -/-
Gr-1
CD11b
46.9
18.7
45.7
16.2
47.9
24.6
47.7
18.8
35.4
41
44.4
18.6
27.3
57.9
53.1
24.4

Figure 4.

Figure 4. Non-hematopoietic cells are sufficient and the main contributors to enhanced bone marrow myeloid cell production upon TLR4 stimulation *in vivo.*

(A) Bar graphs show total cell numbers in the bone marrow (BM) of PBS (white bars) or LPS (grey bars) treated WT, TLR4$^{-/-}$, hematopoietic-TLR4$^{-/-}$, and non-hematopoietic-TLR4$^{-/-}$ chimeric mice. **(B)** Bar graphs show total spleen cell numbers of PBS (white bars) or LPS (grey bars) treated WT, TLR4$^{-/-}$, hematopoietic-TLR4$^{-/-}$, and non-hematopoietic-TLR4$^{-/-}$ chimeric mice. **(C)** Bar graphs show percentages of Gr-1^{high}CD11b$^{lo/+}$ cells in the BM of PBS (white bars) or LPS (grey bars) treated WT, TLR4$^{-/-}$, hematopoietic-TLR4$^{-/-}$ and non-hematopoietic-TLR4$^{-/-}$ chimeric mice. **(D)** Increase in percentages of Gr-1^{low}CD11b $^{lo/+}$ cells in the BM of PBS (white bars) or LPS (grey bars) treated WT, TLR4$^{-/-}$, hematopoietic-TLR4$^{-/-}$ and non-hematopoietic-TLR4$^{-/-}$ chimeric mice. A–D: Mean ± SEM of three independent experiments with each one PBS and two LPS-treated mice per group and experiment are shown. Statistically significant differences are indicated (*p<0.05, **p<0.01, ***p<0.001). **(E)** Representative FACS profile of Gr-1^{low}CD11b $^{lo/+}$ and Gr-1^{high}CD11b $^{lo/+}$ cells in the BM of WT, TLR4$^{-/-}$, hematopoietic-TLR4$^{-/-}$, and non-hematopoietic-TLR4$^{-/-}$ chimeric mice after PBS or LPS injection of analysis in C and D.

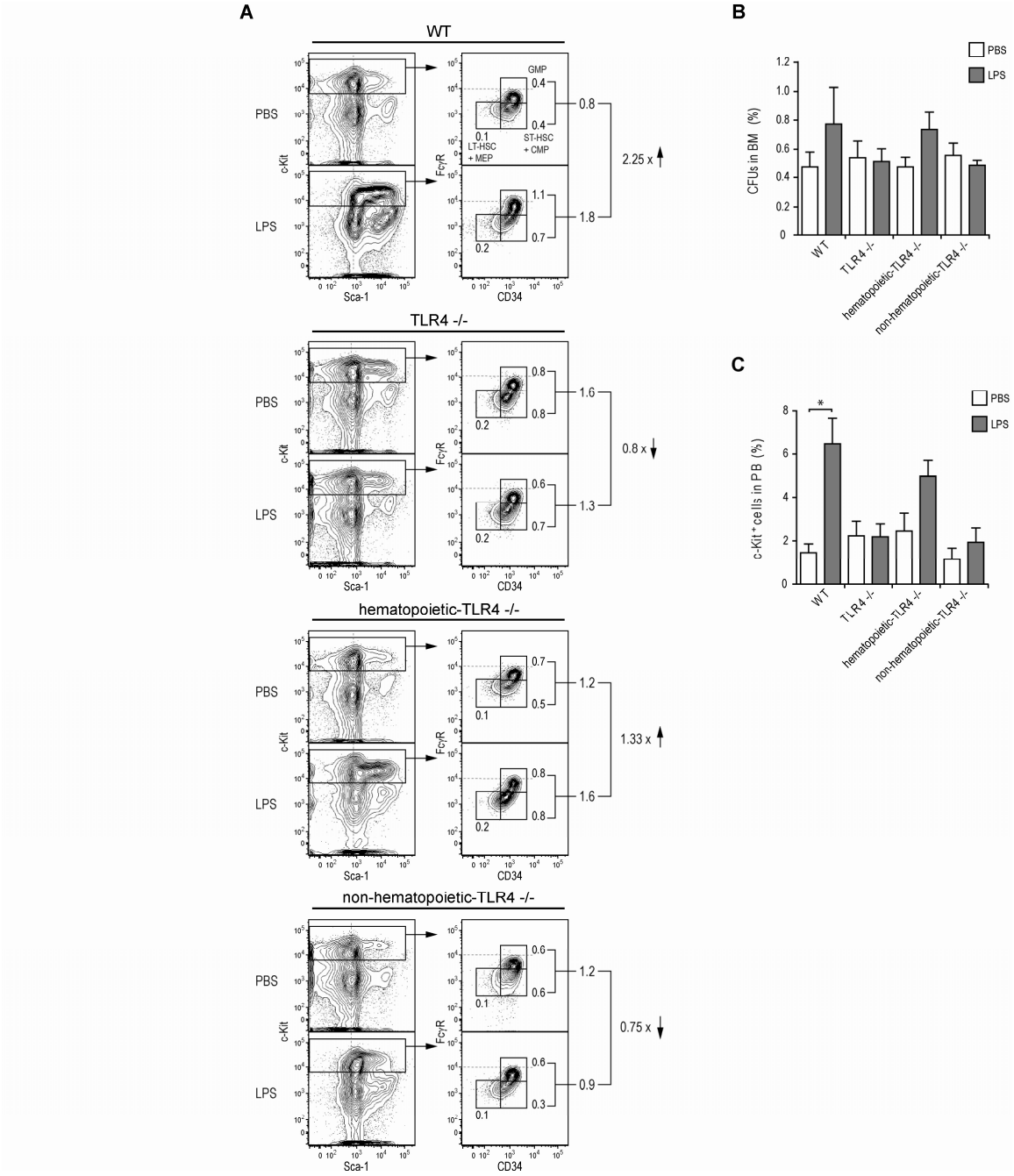
A
WT
PBS
LPS
c-Kit
Sca-1
FcγR
CD34
GMP
0.4
0.4
0.1
LT-HSC
+ MEP
ST-HSC
+ CMP
0.8
1.8
2.25 x
1.1
0.7
0.2
TLR4 -/-
1.6
0.8 x
0.8
0.2
0.6
1.3
0.7
hematopoietic-TLR4 -/-
1.2
1.33 x
0.7
0.5
0.1
1.6
0.8
non-hematopoietic-TLR4 -/-
0.6
0.75 x
0.9
0.3
B
PBS
LPS
CFUs in BM (%)
WT
TLR4 -/-
hematopoietic-TLR4 -/-
non-hematopoietic-TLR4 -/-
C
c-Kit+cells in PB (%)
*

Figure 5.

Figure 5. Non-hematopoietic cells are the main contributors to hematopoietic progenitor cell pattern changes and myeloid CFU increase in bone marrow, and to hematopoietic progenitor cell mobilization upon TLR4 stimulation *in vivo*.

(A) Representative FACS profiles of PI negative, lineage negative BM cells from PBS and LPS injected WT and $TLR4^{-/-}$, and chimeric hematopoietic-$TLR4^{-/-}$ and non-hematopoietic-$TLR4^{-/-}$ mice. Lin^- c-Kit^{high} cells were divided into three subsets based on FcγRII/III and CD34 expression: $CD34^+$FcγRII/III^- (ST-HSC+CMP), $CD34^+$FcγRII/III^+ (GMP), and $CD34^-$FcγRII/III^- (LT-HSC+MEP). Numbers indicate percentage of PI negative cells, and fold-changes in PBS vs. LPS treated mice of combined $CD34^+$ fractions are shown. To better visualize changes in Sca-1 and FcγRII/III expression upon LPS application, dashed lines are drawn. Results are a representative from one out of three independent experiments with each one PBS and two LPS-treated mice per group and experiment. **(B)** Bar graphs show CFU activity (BFU-E, CFU-GM/G/M, CFU-GEMM) of $3x10^4$ BM cells plated per well from PBS (white bars) or LPS (grey bars) injected mice as indicated. Mean ± SEM of three independent experiments with three mice for PBS and four mice for LPS treatment per group are shown. **(C)** Percentage of c-Kit^+ cells in the peripheral blood of PBS (white bars) or LPS (grey bars) treated WT, $TLR4^{-/-}$, hematopoietic-$TLR4^{-/-}$ and non-hematopoietic-$TLR4^{-/-}$ chimeric mice. Mean ± SEM of three independent experiments with three mice for PBS and four mice for LPS treatment per group are shown. Statistically significant differences are indicated (*$p<0.05$).

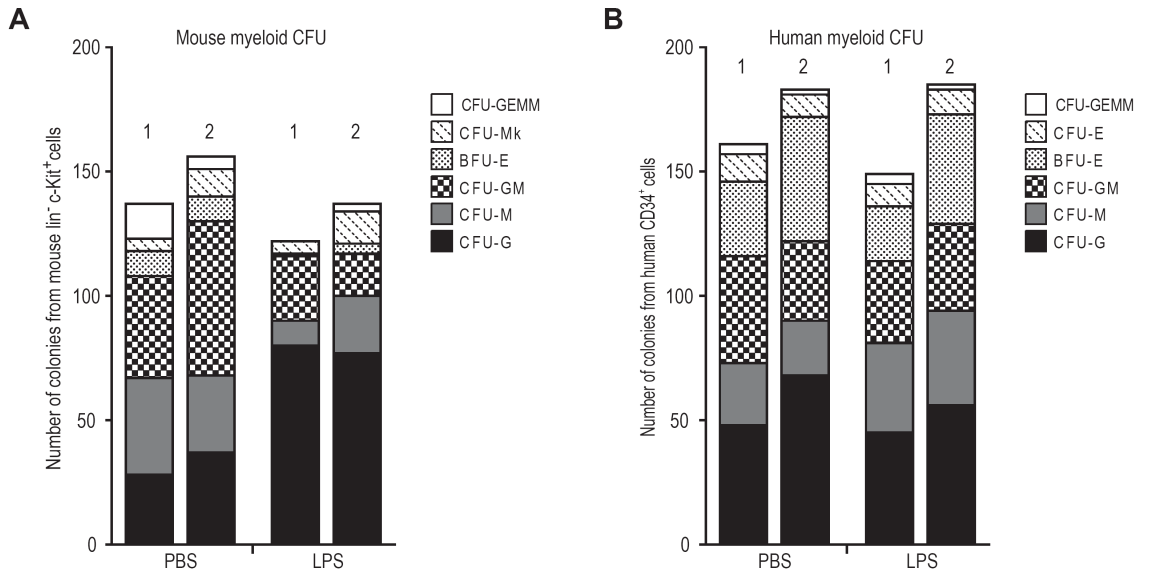

Figure 6. Combined TLR4 and cytokine stimulation of hematopoietic progenitor cells in absence of stroma enhances granulocytic read-out from mouse but not human cells.

(A) Bar graphs show CFU activity of mouse hematopoietic cells after 10 days of cytokine-supplemented culture in absence or presence of LPS (10 μg/ml). $4x10^2$ sorted mouse $lin^-c\text{-}Kit^+$ cells were plated per well and colony numbers were counted from triplicate wells. CFU-GM/G/M, CFU-granulocyte/macrophage, -granulocyte, -macrophage; BFU-E, burst-forming units/erythroid; CFU-Mk, CFU-megakaryocyte; CFU-GEMM, CFU-granulocyte erythrocyte monocyte macrophage. Two independent experiments (indicated with 1, 2) with pooled $lin^-c\text{-}kit^+$ cells from 3 mice are shown. **(B)** CFU activity of human cord blood $CD34^+$ cells after 12 days of cytokine-supplemented culture in absence or presence of LPS (10 μg/ml). One thousand five hundred human $CD34^+$ cells were plated per well and colonies formed were counted in triplicate wells. CFU-E, CFU-erythrocyte. Two independent experiments (indicated with 1, 2) with each $CD34^+$ cord blood cells from different donors are shown.

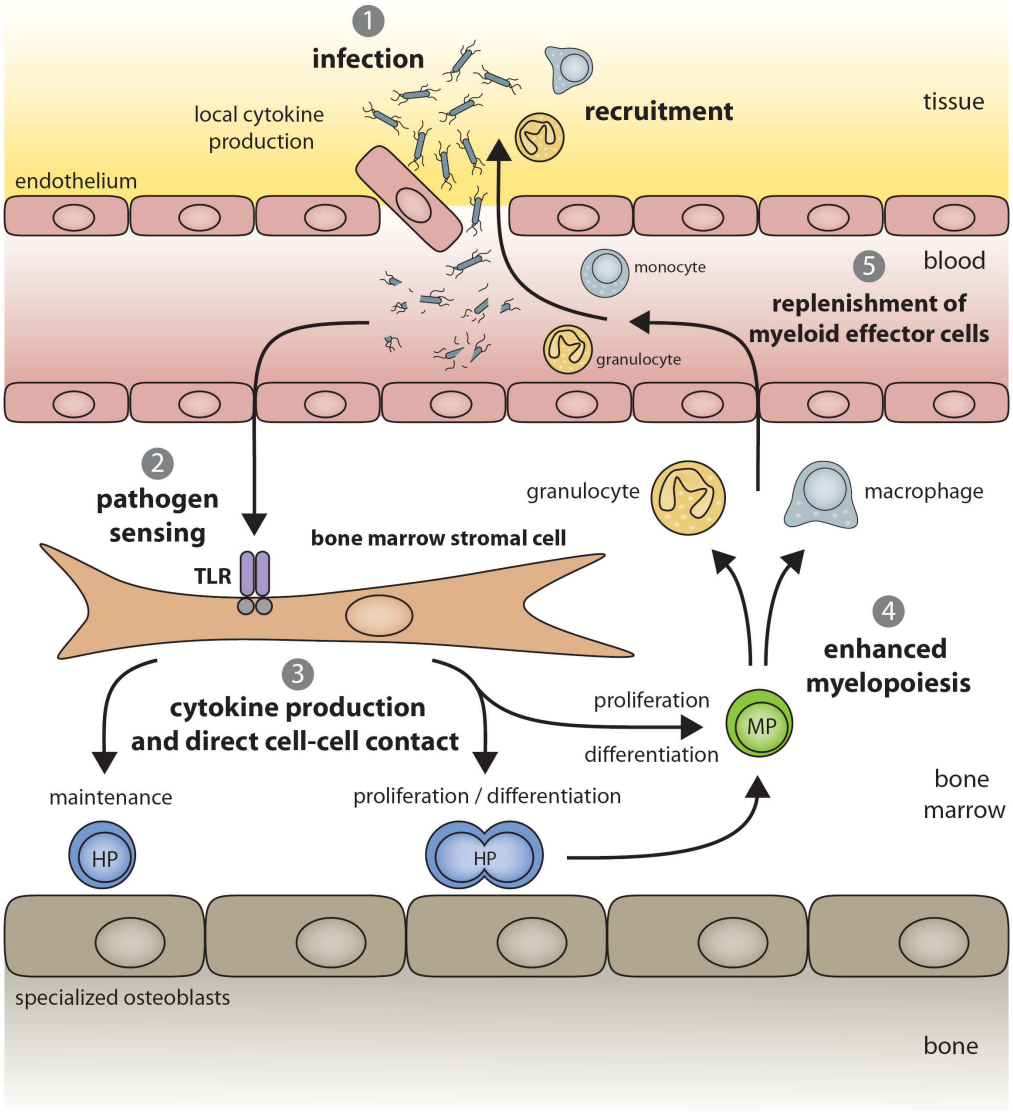

Figure 7. Model for translation of pathogen signals into enhanced production of myeloid effector cells in bone marrow.

During the course of an extended infection bacterial products attain systemic bioavailability and reach the bone marrow (1). BMSCs sense TLR agonists (2) and respond by increasing the production of hematopoietic progenitor (HP) and myeloid progenitor (MP) maintenance and differentiation supporting cytokines (3). As a consequence hematopoietic progenitors are maintained, myeloid differentiation is enhanced (4), and myeloid effector cells are replenished (5).

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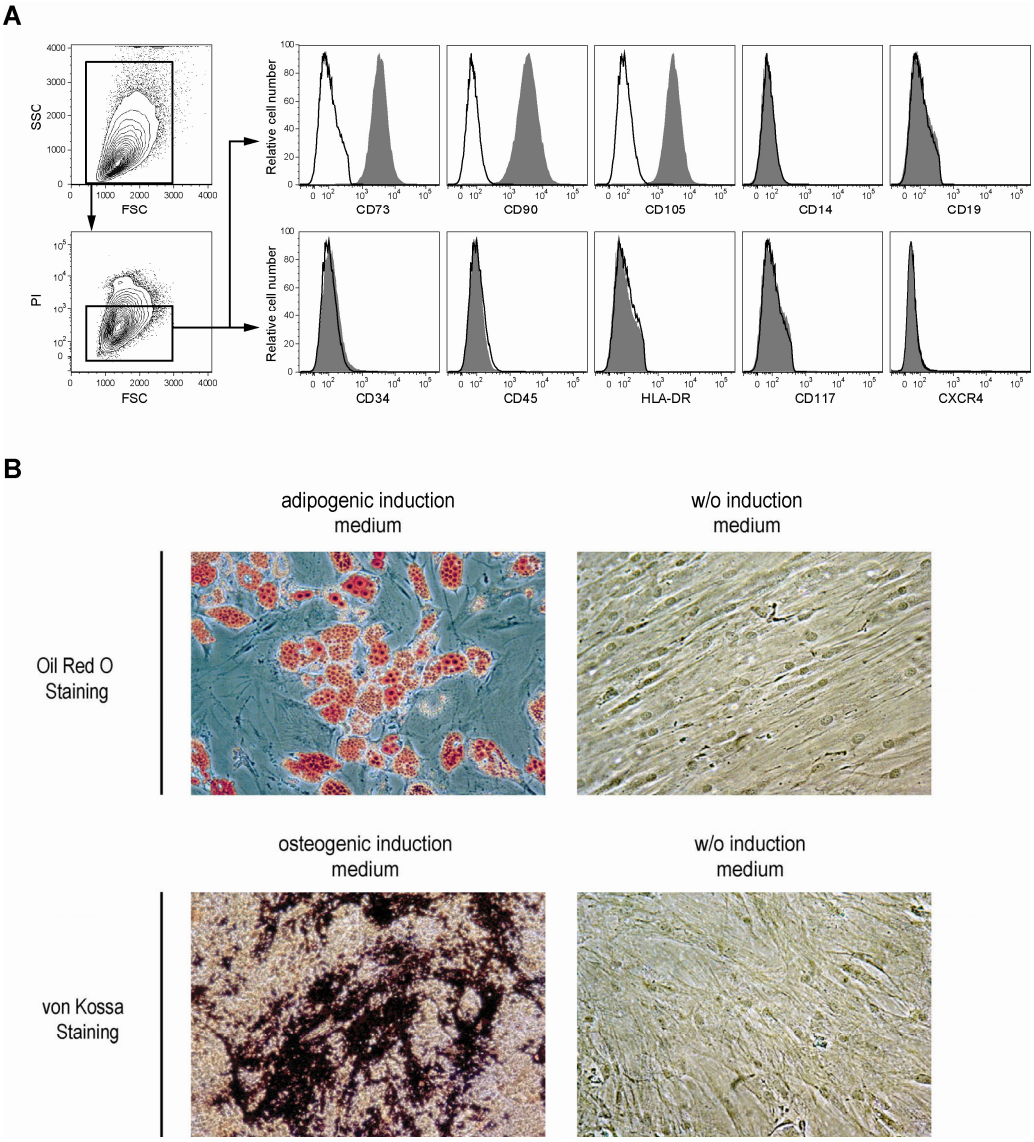
Supplementary material

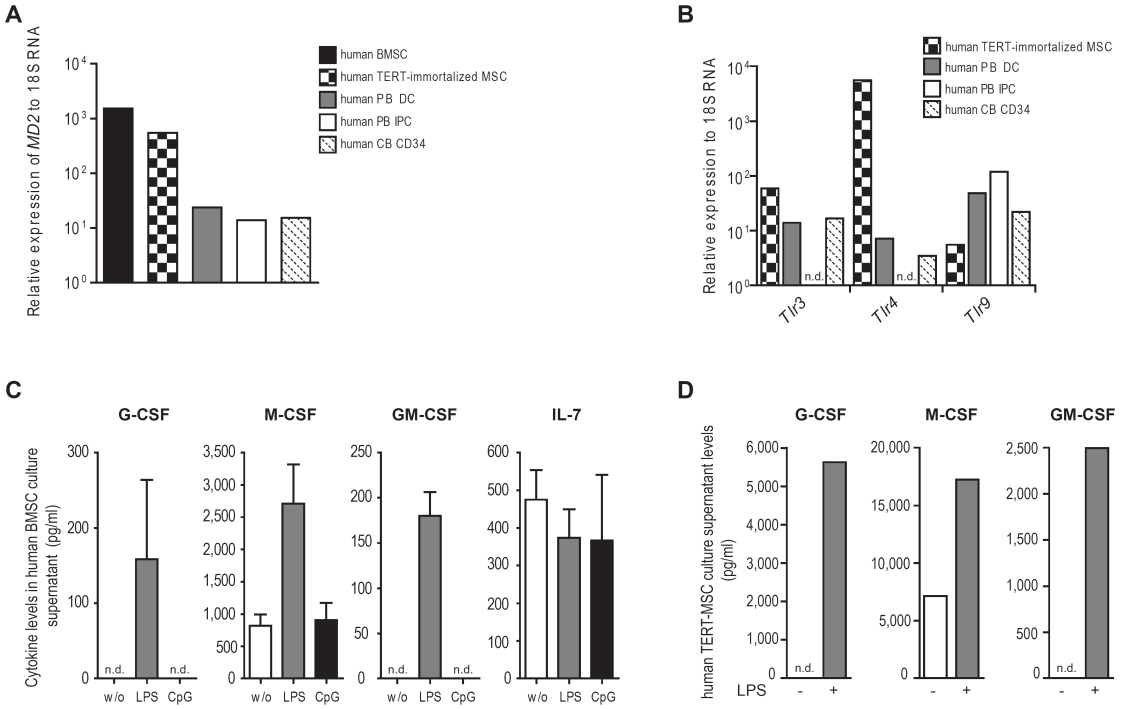
Supplementary Table 1.

Mouse	BMSCs	LPS	Number of cells [1] transplanted	Bone marrow		Thymus
				CD45 [2,3]	CD19 [2,3]	CD45 [2]
1	A	-	$3x10^5$ (12.9)	*NE*	*NE*	*NE*
2	A	-	$3x10^5$ (12.9)	*NE*	*NE*	*NE*
3	A	+	$2x10^5$ (24.3)	*NE*	*NE*	*NE*
4	A	+	$2x10^5$ (24.3)	0.75	0.64	13.9
5	A	+	$2x10^5$ (24.3)	0.14	0.12	1.12
6	B	-	$3x10^5$ (29.3)	*NE*	*NE*	*NE*
7	B	-	$3x10^5$ (29.3)	*NE*	*NE*	*NE*
8	B	-	$3x10^5$ (29.3)	*NE*	*NE*	*NE*
9	B	+	$2x10^5$ (34.9)	0.28	0.23	12.2
10	B	+	$2x10^5$ (34.9)	0.24	0.14	*NE*

Supplementary Table 1. Human cell engraftment in BM and thymus of Rag2$^{-/-}\gamma_c^{-/-}$ mice receiving transplants of human hematopoietic cells co-cultured for 12 days with human BMSCs. ***(1)*** Values represent total hematopoietic cell numbers and percentage of $CD34^+$ cells (in parenthesis) transplanted per animal. Mice were transplanted as newborns and sacrificed at the age of 4 weeks. ***(2)*** Human cell engraftment in the BM ($CD45^+$ and $CD19^+$) and the thymus ($CD45^+$) was determined as a percentage of nucleated cells. ***(3)*** Cells were stained for CD45 FITC first and then column enriched using anti-FITC magnetic beads. If less than 0.1% of enriched nucleated cells were $CD45^+$, mice were considered as not engrafted (*NE* = no engraftment). For co-culture of cells two different BMSC-feeder layers have been used: a primary isolate of BMSCs (A), and TERT-immortalized MSC cell line (B).

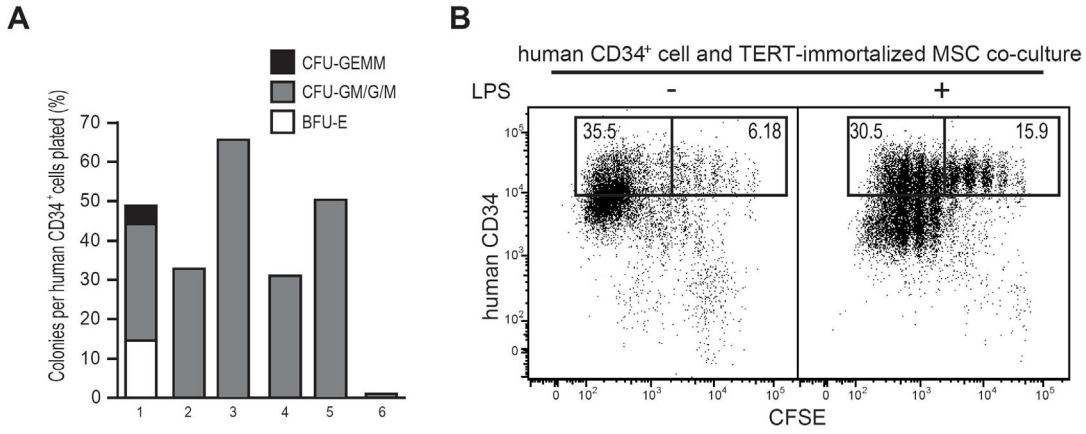


Supplementary Figure S1. Phenotypic and functional characterization of human BMSCs.
(A) Immunophenotype of human BMSCs. Cells were labeled with monoclonal antibodies specific for the molecules indicated (filled histograms) and respective isotype controls (open histograms). One representative analysis out of three independent experiments with different bone marrow donors is shown. **(B)** In vitro differentiation of human BMSCs into adipocytes and osteoblasts. Oil Red O staining of lipid vacuoles was performed 21 days after beginning of stimulation. Von Kossa staining of secreted Ca^{2+} deposits was done 30 days after initiation of stimulation. One representative out of three independent experiments with different bone marrow donors is shown.



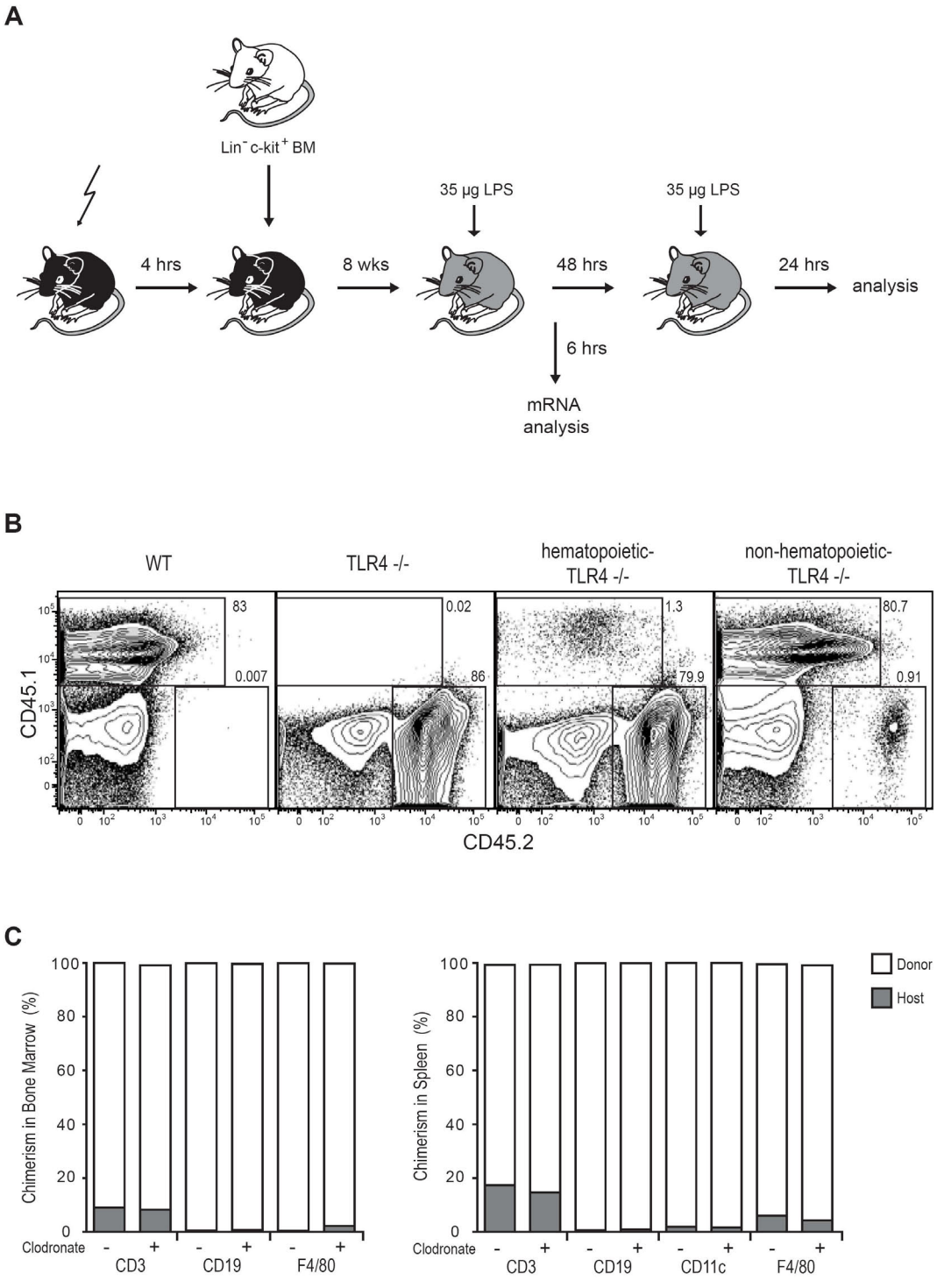
Supplementary Figure S2. *TLR* and *Md-2* expression and effects of TLR4 and TLR9 agonist stimulation on human BMSCs and human TERT-immortalized MSCs.

(A) *Md-2* expression in BMSCs (black bars), TERT-immortalized MSCs (speckled bars), peripheral blood DCs (PB DC, BDCA-1$^+$, CD14$^-$, CD19$^-$, grey bars), peripheral blood IPCs (PB IPC, BDCA-4$^+$, CD14$^-$, CD19$^-$, white bars), and sorted CB CD34$^+$ cells (CD34$^+$, PI$^-$, striped bars). Expression levels are given as arbitrary units relative to endogenous 18S RNA. One representative experiment out of two with different bone marrow, cord blood, and buffy coat donors, and one representative analysis out of two with human TERT-immortalized MSCs is shown. **(B)** *Tlr3, 4,* and *9* expression in TERT-immortalized MSCs (speckled bars) compared to peripheral blood DCs (PB DC, BDCA-1$^+$, CD14$^-$, CD19$^-$, grey bars), peripheral blood IPCs (BDCA-4$^+$, CD14$^-$, CD19$^-$, white bars), and sorted CB CD34$^+$ cells (CD34$^+$, PI$^-$, striped bars). Expression levels are given as arbitrary units relative to endogenous 18S RNA. One representative experiment out of two with different cord blood and buffy coat donors, and one representative analysis out of two with human TERT-immortalized MSCs is shown. **(C)** Bar graphs show protein levels of G-CSF, M-CSF, GM-CSF, and IL-7 in supernatants taken from un-stimulated (white bars) and either LPS stimulated (10 µg/ml, grey bars) or CpG stimulated (1 µM, black bars) human BMSC cultures. Mean ± SEM of three independent experiments with BMSCs from different bone marrow donors are shown. n.d., not detectable. **(D)** G-CSF, M-CSF and GM-CSF protein levels in culture supernatants of LPS (grey bars) versus un-stimulated (white bars) human TERT-immortalized MSC. n.d., not detectable within the sensitivity of the assay. One representative out of two experiments is shown.



Supplementary Figure S3. Myeolopoiesis and hematopoietic progenitor maintenance supporting activity of human TERT-immortalized MSCs.

(A) Myeloid colony-forming activity of sorted human CB PI^- $CD34^+$ cells stimulated with SCF, Flt3L, TPO, IL-3, IL-6, IL-11, GM-CSF, and EPO (1), with SCF, Flt3L, TPO, and supernatants from un-stimulated (2) and LPS stimulated TERT-immortalized MSCs (3), and respective controls: un-stimulated TERT-immortalized MSC supernatant with addition of SCF, Flt3L, TPO and LPS (4); un-stimulated TERT-immortalized MSC supernatant with addition of SCF, Flt3L, TPO, and 10ng/ml M-CSF, 900pg/ml G-CSF, and 300 pg/ml GM-CSF (5); only SCF, Flt3L, TPO and LPS added (6). One representative out of two experiments with different $CD34^+$ cord blood cells and different TERT-immortalized MSC supernatants is shown. **(B)** FACS plots show division of human $CD34^+$ cord blood cells by dilution of CFSE at day 12 of culture on human TERT-immortalized MSCs with or without addition of LPS (10 μg/ml) as indicated. Gates indicate ≤3 and ≥4 times divided human $CD34^+$ cells. Results from one representative out of three independent experiments with different cord blood donors are shown.



Supplementary Figure S4.

Supplementary Figure S4. Generation and LPS treatment of WT, TLR4$^{-/-}$, hematopoietic-TLR4$^{-/-}$ and non-hematopoietic-TLR4$^{-/-}$ chimeric mice.

(A) For the analysis of *G-csf* mRNA expression mice received a single injection of LPS (35μg) and were sacrificed 6 hours later. For the analysis of cellular subsets, CFU activity and plasma G-CSF levels, mice received two LPS injections (35μg) in a 48 hour interval. Mice were sacrificed 24 hours after the second injection. **(B)** Representative FACS analysis of CD45.1 and CD45.2 congenic marker expression in the BM of age and sex matched WT, TLR4$^{-/-}$ and transplanted chimeric hematopoietic-TLR4$^{-/-}$ and non-hematopoietic-TLR4$^{-/-}$ mice 8 weeks after transplantation. One representative out of three independent experiments with three mice in each group is shown. **(C)** Bar graphs show percentage of chimerism in bone marrow and spleen of one untreated and one clodronate-treated hematopoietic-TLR4$^{-/-}$ chimeric animal 8 weeks after transplantation. Based on FACS analysis using CD45.1 and CD 45.2 congenic marker expression together with monoclonal antibodies directed against the molecules indicated respresentative for T cells (CD3^{+}), B cells (CD19^{+}), and macrophages (F4/80^{+}, CD11c^{-}), donor-derived cells were distinguished from host-derived ones. Representative analysis of n=10 mice not pretreated with clodronate and n=2 mice pretreated with clodronate is shown.

The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis

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Dendritic cell (DC) development is efficiently supported by Flt3-ligand or GM-CSF in vitro, and lymphoid-organ DC maintenance in vivo is critically dependent on Flt3-ligand. However, the relevance of GM-CSF for lymphoid-tissue DC maintenance and the importance of both cytokines for nonlymphoid organ DC homeostasis are not defined. Here, we show that, although *Gm-csfr* and *Flt3* are both expressed in DC progenitors, *Gm-csfr* is expressed predominantly in monocytes, classical DCs (cDCs), and skin DCs, whereas *Flt3* is expressed in both cDCs and plasmacytoid DCs (pDCs). In accordance with the respective cytokine receptor expression, DC progenitor and pDC numbers are primarily affected by Flt3-ligand deficiency, whereas both splenic and lymph node cDCs and dermal DCs are reduced in the absence of either GM-CSF or Flt3-ligand. Combined lack of GM-CSF and Flt3-ligand in newly generated double-deficient mice leads to further significant reductions of DC progenitors and dermal DCs. In line with the decrease of respective DC subsets, T-cell and antigen-specific IgG responses decline progressively, from wild-type to GM-CSF– to Flt3-ligand– to double-deficient mice, upon subcutaneous antigen delivery. These data thus show the concerted action of GM-CSF and Flt3-ligand on DC homeostasis in vivo. (Blood. 2009;114: 835-843)

Introduction

Steady-state maintenance of tolerance and induction of the adaptive immune response during infection and inflammatio both require the specialized functions of dendritic cells (DCs). Although the role of DCs in immune regulation is crucial, they represent a very small fraction of short-lived cells of the hematopoietic system distributed throughout the body with particularly high concentrations at environmental interfaces and in lymphoid organs. DCs can be divided into multiple subsets based on location, function, and surface markers. Here, we divide them into plasmacytoid DCs (pDC) that are uniquely equipped to produce type I interferons during infection, lymphoid-tissue resident DCs (also called classical DCs; cDCs), and nonlymphoid tissue, migratory DCs such as epidermal DCs (Langerhans cells; LCs) and dermal DCs that are, in contrast to pDCs, more efficien in extracellular antigen uptake, presentation, and activation of lymphocytes.[1-3]

DCs can efficientl be differentiated in vitro by stimulating monocytes or hematopoietic progenitors with granulocyte macrophage colony-stimulating factor (GM-CSF).[4,5] At the same time, GM-CSF inhibits in vitro pDC development through activation of STAT5 signaling.[6-8] Surprisingly, mice lacking GM-CSF or its receptor had only small decreases in lymphoid-organ DCs with a maximum reduction of 3-fold in lymph node cDCs and only a modest reduction of LCs, whereas GM-CSF transgenic mice showed similar opposite effects.[9,10] Thus, at least in the presence of compensatory cytokines, GM-CSF seemed to add little to steady-state DC maintenance, and it was suggested that GM-CSF mostly contributes to inflammator DC generation, potentially from monocytes, in vivo. Indeed, it was recently shown that adoptively transferred monocytes only generate DCs in nonlymphoid tissue and spleen in an inflammator environment (eg Naik et al[11] and Varol et al[12]), and bone marrow–derived DCs cultured in GM-CSF represent tumor necrosis factor-α– and inducible nitric oxide synthase–producing inflammator DCs observed in vivo.[13]

In contrast, Flt3-ligand (FL) supports the in vitro differentiation of progenitor cells, but not monocytes, into both cDCs and pDCs[7,14] and genetic deletion of FL or treatment of mice with Flt3 (fms-related tyrosine kinase 3; Flk2) inhibitors leads to a 10-fold reduction of lymphoid-organ pDCs and cDCs,[15,16] whereas LCs are little or not affected.[9] In addition, FL injection or overexpression of FL results in the expansion of both pDCs and DCs in all lymphoid and nonlymphoid organs.[17-20] In line with this, it has been shown that DC development is confine to hematopoietic precursors in the bone marrow expressing Flt3,[18,21] that Flt3 signaling can also instruct Flt3-negative precursors to differentiate into both pDCs and cDCs,[22] and that FL is involved in all lymphoid-organ DC development and expansion from early progenitors in the bone marrow to immediate DC progenitors in lymphoid tissues.[18,23] Moreover, early progenitors such as "macrophage and DC progenitors" (MDPs)[24] and "MDP$^{\Delta}$"[23] that give rise to monocytes, macrophages, and DCs, and probably further downstream[25,26] "common DC progenitors" (CDPs)[27] and "pro-DCs,"[28] that give rise solely to pDCs and DCs, have recently been identified All express c-kit (CD117, the receptor for stem cell factor) and Flt3 (CD135), but no mature lineage marker.

To further elucidate the roles of GM-CSF and FL on DC homeostasis, we here systematically compared GM-CSF and Flt3 receptor expression on ex vivo–isolated DC progenitors, monocytes, and lymphoid and nonlymphoid tissue DC populations, and

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tested the effect of in vivo GM-CSF, FL, and combined GM-CSF and FL deficienc on lymphoid and nonlymphoid organ DCs at steady-state and on immune responses upon vaccination.

Methods

Mice

GM-CSF$^{-/-}$ mice were obtained from Dr J.A. Whitsett (Hospital Medical Center, Cincinnati, OH),[29] FL$^{-/-}$ mice were obtained from Dr J.J. Peschon (Immunex Corporation, Seattle, WA).[15] GM-CSF$^{-/-}$ FL$^{-/-}$ double-knockout (DKO) mice were generated by crossbreeding GM-CSF$^{-/-}$ and FL$^{-/-}$ mice. All knockout mice were on the C57BL/6 background. C57BL/6 mice were used as wild-type (WT) controls. Sex- and age-matched, 6- to 12-week-old mice were used in the studies. All mice were bred and maintained at the Institute for Research in Biomedicine animal facility. Mice were treated in accordance with guidelines of the Swiss Federal Veterinary Office and experiments were approved by the Dipartimento della Sanità e Socialità.

Antibodies

All antibodies were purchased from eBiosciences, unless otherwise stated. The following monoclonal antibodies conjugated to different fluorochromes or biotin were used: CD3ε (145-2C11), c-kit (ACK2), M-CSFR (AFS98), Flt3 (A2F10), IL7Rα (A7R34), MHCII (M5/114.15.2), CD11c (N418), B220 (RA3-6B2), CD45RA (14.8; Becton Dickinson), CD40 (3/23; Becton Dickinson), Gr-1 (RB6-8C5), CD11b (M1/70), NK1.1 (PK136), CD19 (MB19-1 and ID3; Becton Dickinson), CD45 (30-F11; Becton Dickinson), CD45.1 (A20), CD45.2 (104), CD4 (RM4-5), CD8α (53-6.7), and Ter119 (Ter119). Biotinylated antibodies were visualized with streptavidin–fluorescein isothiocyanate (FITC), streptavidin-APC or streptavidin-APC-Cy7.

Cell preparation and flow cytometry

Cells isolated from organs were analyzed by flow cytometry or sorted as previously described.[16,23,24,27,30] Dead cells were excluded by propidium iodide staining. Cells were acquired on a FACSCanto or sorted with a FACSAria (Becton Dickinson) and analyzed with the use of FlowJo software (TreeStar).

Quantitative reverse transcription–polymerase chain reaction analysis

Cells were sorted and resuspended in TRIzol LS reagent (Invitrogen). RNA was extracted, followed by DNase I treatment with the use of the DNA-free kit (Applied Biosystems). Equal amounts of RNA were used for cDNA synthesis and real-time polymerase chain reaction (PCR), which was performed and analyzed as previously described.[27] Taqman probes for *18S* (HS99999901_s1), mouse *Gm-csfr* (*Csf2ra*; Mm00438331_g1), *Flt3* (Mm00438996_m1), and *M-csfr* (Mm00432689_m1) were purchased from Applied Biosystems. Results were normalized to *18S*.

Immunofluorescence

Epidermal sheets were separated from the dermis with the use of 0.5 M ammonium thiocyanate (Sigma-Aldrich) at 37°C for 30 minutes, stained, and prepared on slides. Images were taken on a Nikon Eclipse E800 microscope with a CCD Qimaging camera with the use of OpenLab software. All images were acquired with the use of a Nikon Plan Apo 20×/0.75 numeric aperture (NA) objective lens.

In vivo T-cell proliferation assay

Naive OT-II T cells (CD4$^+$CD8$^-$CD25$^-$CD44^{lo}CD62L^{hi}) were sorted from spleens and lymph nodes (LNs) of OT-II/RAG1$^{-/-}$ transgenic mice. Cells were labeled with 2.5 μM CFSE (carboxyfluorescein diacetate succinimidyl ester; Invitrogen), and 6×10^4 cells were injected intravenously into mice. Sixteen hours later, mice were immunized with 2 μg whole OVA protein (Sigma-Aldrich) and 4 μg monophosphoryl lipid A (InvivoGen) in the flank. Draining LNs were analyzed by flow cytometry after 3 days.

Antibody response

Mice were immunized in the footpads as described in the T-cell proliferation assay. A booster immunization was given 3 weeks later. Serum was collected and analyzed by enzyme-linked immunoabsorbent assay (ELISA). Plates were coated with 10 μg/mL OVA and blocked with 2% BSA in PBS, and serial dilutions of sera from immunized mice were added. OVA-specific antibodies were detected with alkaline phosphatase–conjugated goat anti–mouse antibodies (Southern Biotech) and pNPP substrate (Sigma-Aldrich). Plates were read at 405 nm with a microplate reader (Molecular Devices). Pooled sera from hyperimmunized mice were used as a standard to calculate relative units.

Statistics

Data were analyzed by Prism 4 (GraphPad Software) with the use of the nonparametric unpaired Mann-Whitney U test. Graphs show the mean plus or minus SEM. *P* was considered significant at values less than .05.

Results

Cell-specific expression of *Flt3*, *Gm-csfr*, and *M-csfr*

We tested the relative mRNA expression of *Flt3* and *Gm-csfr* between ex vivo–isolated progenitor cells, subpopulations of monocytes, and lymphoid and nonlymphoid organ DCs with the use of quantitative real-time PCR. Cells were sorted from bone marrow, spleen, lymph nodes, epidermis, and dermis.

In the earliest bone marrow progenitor population analyzed, c-kit^{hi} cells, which contain hematopoietic stem cells, as well as multiple early lineage-restricted progenitors, *Flt3* and *Gm-csfr* expression were relatively low, whereas expression increased slightly on MDP$^{\Delta}$s and even more so on CDPs (Figure 1A-B; progenitor population and sorting gate define in supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).[23,27] A comparative back-gating analysis of MDP$^{\Delta}$ and CDP showed that approximately two-thirds of CDPs are contained within the MDP$^{\Delta}$ gate, whereas the MDP$^{\Delta}$ gate includes, with respect to c-kit and Flt3 expression, a heterogeneous population of cells (supplemental Figure 1).

In both the spleen and lymph node, cDCs and pDCs expressed *Flt3* at high levels, and similar levels were also observed in CD40^{hi}CD11c^{int} cells that include both LCs and dermal DCs that have trafficked to the lymph node from the skin.[31] Conversely, LCs and dermal-derived DCs, which were sorted directly from the skin, had much lower levels of *Flt3* expression compared with CD40^{hi}CD11c^{int} cells. *Flt3* levels were also very low or absent in either Gr1$^+$ or Gr1$^-$ monocytes isolated from the blood, spleen, and bone marrow (BM; Figure 1A).[32]

As expected, given their responsiveness to GM-CSF in culture, all sorted monocytes expressed high levels of *Gm-csfr*. Furthermore, skin-derived DCs and cDCs had similarly high levels of *Gm-csfr* gene expression. As in the case with *Flt3*, *Gm-csfr* expression levels reversed in the migratory skin DCs isolated from skin-draining LNs compared with DCs isolated directly from the skin, suggesting that upon maturation and migration, skin-derived DCs down-regulate *Gm-csfr* expression and at the same time up-regulate *Flt3* expression. In contrast to cDCs, pDCs expressed relatively low levels of *Gm-csfr* (Figure 1B).

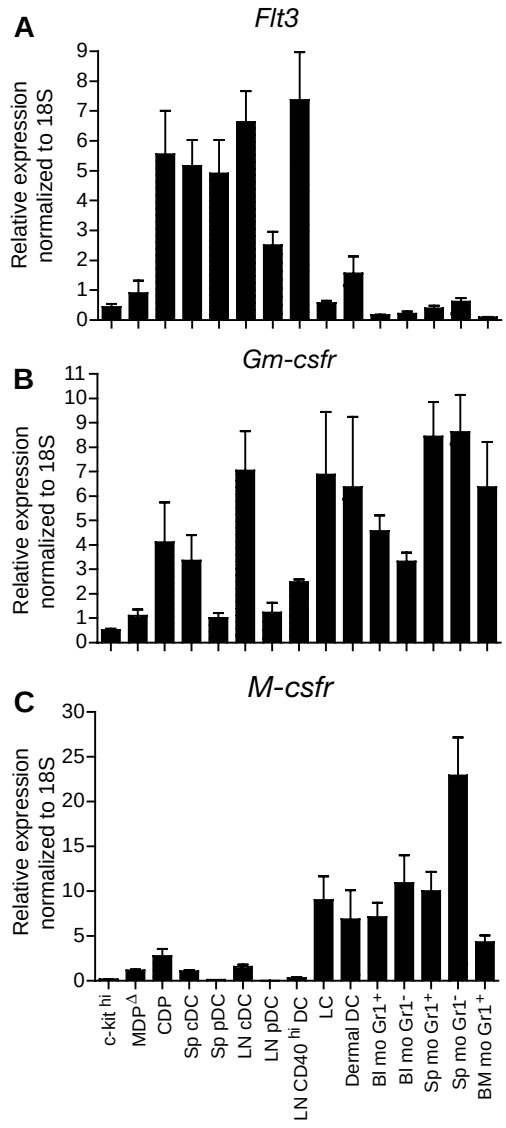

Figure 1. *Flt3*, *Gm-csfr*, and *M-csfr* mRNA expression in progenitor and myeloid cells. Real-time PCR analysis of mRNA expression of *Flt3* (A), *Gm-csfr* (B), and *M-csfr* (*C*) in cell populations sorted from WT mice based on the following markers: c-kit^{hi} (lin$^-$c-kit^{hi}); MDP$^{\Delta}$ (lin$^-$M-CSFR^{hi}); CDP (lin$^-$c-kit^{int}Flt3$^+$IL7Rα^-); spleen (Sp) and LN cDC (CD19$^-$MHCII$^+$CD11c$^+$) and pDC (CD19$^-$CD45RA$^+$CD11c$^+$); LN CD40^{hi} DC (CD19$^-$CD40^{hi}CD11c^{int}); Langerhans cells (LC from epidermis) and dermal DC (MHCII$^+$CD45$^+$); blood (Bl), spleen, and bone marrow (BM) monocytes (mo) (M-CSFR$^+$CD11b$^+$ and Gr-1$^+$ or Gr-1$^-$). Data shown are averages of 3 independent experiments.

In addition to Flt3 and GM-CSF signaling in DC development, recent studies have suggested a role for macrophage colony-stimulating factor receptor (M-CSFR, CSF1-R) ligands in DC development. As shown by M-CSFR reporter mice, *M-csfr* mRNA is expressed by most lymphoid organ DCs,[33] and DC progenitors, such as MDP$^{\Delta}$s and CDPs, are sorted based on their M-CSF receptor expression.[23,27] Furthermore, M-CSFR–deficien mice have slightly reduced pDC and cDC numbers,[33] M-CSFR is required for in vivo LC development,[30] and M-CSFR was also shown to support pDC and cDC development in vitro and in vivo.[27,34]

We therefore analyzed the mRNA expression levels of *M-csfr* in all respective populations. Although both monocyte subsets and progenitors were sorted on the basis of high surface M-CSFR expression, the mRNA levels in all monocyte subsets tested were much higher than in MDP$^{\Delta}$s or CDPs. Similar high levels of *M-csfr* mRNA were detected in LCs and dermal DCs compared with monocytes. The LC data are in agreement with the findin that LCs develop from a monocyte precursor and require M-CSFR for development.[30] Only cDCs in the spleen and LN expressed detectable *M-csfr*, whereas *M-csfr* expression was very low or absent in pDCs and in CD40^{hi}CD11c^{int} cells (Figure 1C).

Generation of GM-CSF and FL DKO mice

To test the effect of the combined absence of GM-CSF and FL on DC homeostasis in vivo, we generated GM-CSF and FL DKO mice by cross-breeding the 2 single-knockout mice. The lack of gene sequences for *Flt3l* and *Gm-csf* and the absence of respective serum cytokines were confirmed Interestingly, no significan compensatory increase of FL, GM-CSF, or M-CSF was detected in serum from any of the cytokine-deficien animals (supplemental Figure 2).

Absence of GM-CSF and FL reduces DC progenitors in the BM

To determine the effects of GM-CSF and FL on DC progenitors, BM cells from GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice were analyzed. Cells were firs gated on those lacking the following lineage markers: B220, CD11b, CD19, CD3ϵ, CD4, CD8α, Gr-1, Ter119, and NK1.1 (Figure 2A,C firs row). There was a significan reduction of approximately one-third in the absolute numbers of lineage-negative cells in FL$^{-/-}$ and DKO mice compared with WT mice. When cells were further subdivided into MDP$^{\Delta}$ (M-CSFR^{hi}) and CDP (c-kit^{int}Flt3$^+$M-CSFR$^+$IL7Rα^-) populations (supplemental Figure 1), FL$^{-/-}$ mice had 2-fold fewer MDP$^{\Delta}$s and CDPs compared with WT mice at 6 to 9 weeks of age, a findin that was significan for both absolute and relative reductions (Figure 2B-C; Table 1; supplemental Tables 1-2). This findin parallels the decreased myeloid colony-forming unit (CFU) activity measured from the BM of FL$^{-/-}$ mice.[15]

Although in GM-CSF$^{-/-}$ mice the total numbers of both MDP$^{\Delta}$s and CDPs seemed slightly reduced, this reduction was not statistically significan with the number of animals analyzed (Figure 2B-C). In DKO mice, however, a synergistic effect through the absence of GM-CSF and FL was observed, with 7.1-fold and 4.6-fold lower numbers of MDP$^{\Delta}$s and CDPs, respectively, compared with WT mice. These results were significan for both absolute and relative reductions (Figure 2B-C; Table 1; supplemental Tables 1-2). Therefore, macrophage and DC-specifi progenitors express *Flt3* and *Gm-csfr* mRNA (Figure 1), and both cytokines are critical for their differentiation and/or maintenance in steady-state.

Although the Flt3$^+$ fraction of the common lymphoid progenitor (CLP; c-kit^{int}Flt3$^+$M-CSFR$^{lo/-}$IL7Rα^+) was unchanged in GM-CSF–deficien mice, FL$^{-/-}$ mice, as previously shown for mice deficien in Flt3, had significantl lower numbers of CLPs, and a further significan reduction of absolute CLP numbers was observed in DKO mice (Figure 2B-C).[15,35] However, relative CLP frequencies were nearly identical between FL$^{-/-}$ and DKO mice (0.028% ± 0.003% and 0.027% ± 0.003% of total nucleated cells, respectively; data not shown).

Effect of GM-CSF and FL on DC subsets in steady state

Spleens of WT, GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice were analyzed in steady state for leukocyte subsets. FL$^{-/-}$ and DKO mice had on average a slightly lower spleen cellularity compared with WT or GM-CSF$^{-/-}$ mice (Figure 3A). Mice lacking GM-CSF, FL, or both

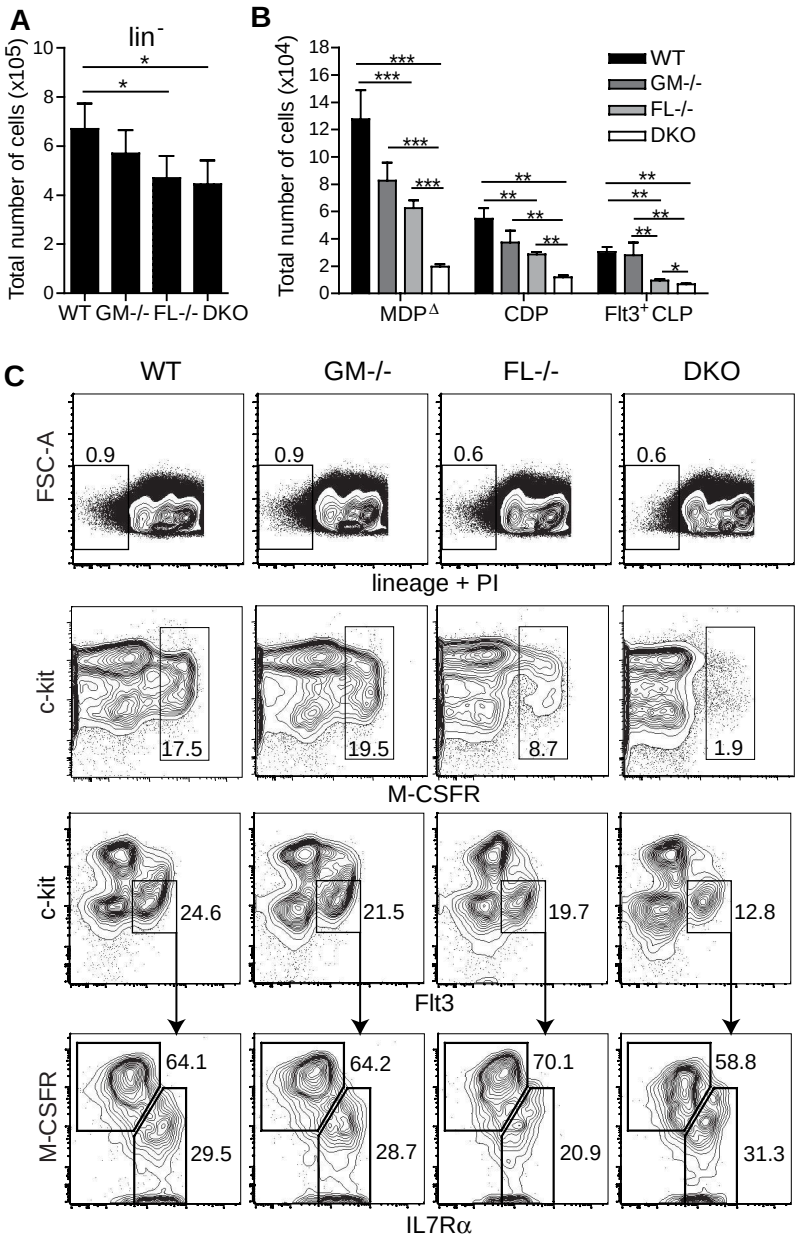

Figure 2. Dendritic cell progenitors are significantly reduced in the absence of GM-CSF and FL. Quantification of total cell numbers of lineage-negative (lin$^-$) cells (A) and for each progenitor population (B) (n = 5-7 mice/group). *$P < .05$; **$P < .01$; ***$P < .001$. (C) Representative fluorescence-activated cell sorting (FACS) plots of stained BM cells from WT, GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice. Gating of lin$^-$ cells (dead cells excluded by PI staining; first row). Second and third rows were first gated on lin$^-$ cells. MDP$^\Delta$ gate: M-CSFR^{hi} (second row). CDP and Flt3$^+$ CLP gates were as follows: CDP, c-kit^{int}Flt3$^+$M-CSFR$^+$IL7Rα^-; Flt3$^+$ CLP, c-kit^{int}Flt3$^+$M-CSFR$^{lo/-}$IL7Rα^+ (third and fourth rows). Numbers in or beside boxes indicate percentage of cells shown.

Table 1. Relative and absolute fold reductions of progenitor cells and DCs in knockout mice compared with WT mice

Cell population	GM-CSF$^{-/-}$	FL$^{-/-}$	DKO
MDP$^\Delta$	1.0/1.5	1.3*/2.3*	2.9*/7.1*
CDP	1.0/1.5	1.3*/1.9*	2.4*/4.6*
Spleen cDC	1.7*/1.8	5.7*/7.5*	4.9*/7.0*
Spleen pDC	1.2/0.8	6.2*/5.6*	6.7*/9.9*
LN cDC	3.4*/4.0*	28*/8.3*	7.5*/41.6*
LN pDC	1.4/1.8	10.9*/7.5*	19.4*/79*
LN CD40^{hi}CD11c^{int} DC	1.8/2.3	1.5/2.3	3.3*/8.3*
CD45$^+$MHCII$^+$ dermal DC	1.4*/ND	2.2*/ND	4.8*/ND
LC	ND/2.0*	ND/1.3*	ND/1.5*

Values are the mean relative fold reduction/absolute fold reduction. DKO indicates double knockout; and ND, not determined.

*Significant reduction compared with WT ($P < .05$).

cytokines had no significan reduction in the percentages of B cells (CD19$^+$) or T cells (CD3$^+$; Figure 3B). Reductions in both Gr1$^+$ and Gr1$^-$ subsets of monocytes (M-CSFR$^+$CD11b$^+$) were equally present in GM-CSF$^{-/-}$ and DKO mice, suggesting, as expected, little involvement of FL in steady-state monocyte homeostasis (Figure 3C; supplemental Figure 3). As described, the percentage of natural killer (NK) cells (CD3$^-$NK1.1$^+$) was significantl reduced in FL$^{-/-}$ compared with WT or GM-CSF$^{-/-}$ mice,[15] and DKO mice exhibited a similar percentage of NK1.1$^+$ cells as FL$^{-/-}$ mice (Figure 3C).

Analysis of spleen cDCs (MHCII$^+$CD11c$^+$) showed a signif - cant 1.7-fold reduction in GM-CSF$^{-/-}$ mice compared with WT mice. FL deficienc had an even greater effect, with an average 5.7-fold relative decrease (7.5-fold reduction in absolute cDC

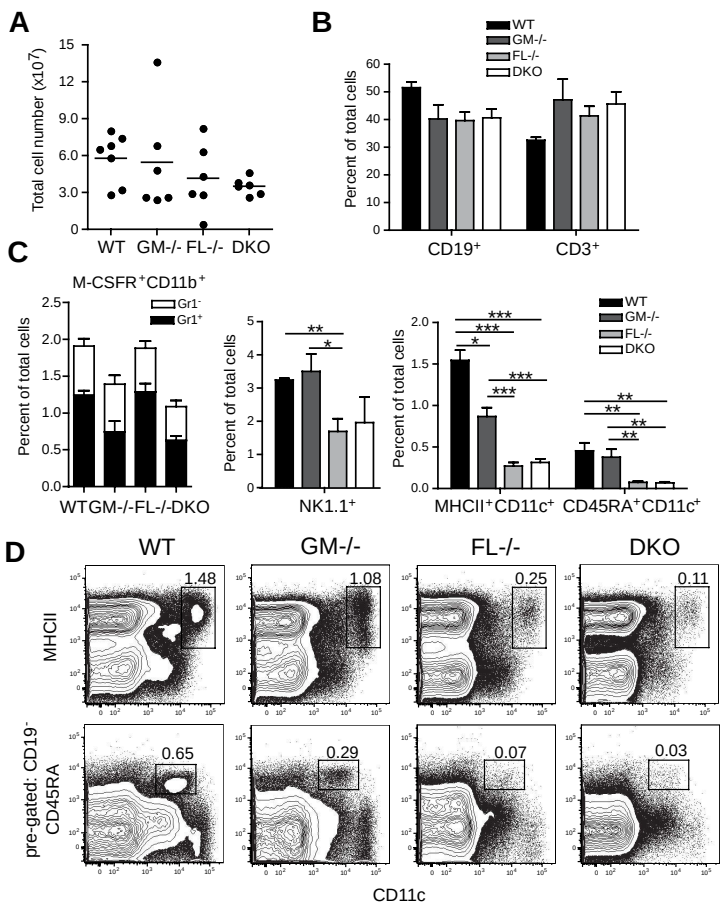

Figure 3. Analysis of cell subsets in the spleen of DKO mice in steady state. (A) Total cellularity of spleens from WT, GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice. Horizontal lines indicate mean values of the results. (B) Percentage of CD19$^+$ and CD3$^+$ cells in the spleen. (C) Percentage of myeloid cell subsets: Monocytes were first gated as M-CSFR$^+$CD11b$^+$ and separated into Gr1$^+$ or Gr1$^-$ cells; NK cells were gated as CD3$^-$NK1.1$^+$; DCs were divided into cDCs (MHCII$^+$CD11c$^+$) and pDCs (CD19$^-$CD45RA$^+$CD11c$^+$) with representative FACS plots shown (D). CD45RA versus CD11c plots were first gated on CD19$^-$ cells. Numbers in FACS plots are percentages of total nucleated cells. *$P < .05$; **$P < .01$; ***$P < .001$.

numbers). Interestingly, mice lacking both GM-CSF and FL had no further reduction of cDCs compared with FL-only deficien mice (Figure 3C-D; Table 1; supplemental Tables 1-2). In addition, CD8α$^-$ and CD8α$^+$ cDCs were equally reduced in both FL$^{-/-}$ and DKO mice (data not shown). GM-CSF$^{-/-}$ and WT mice had similar frequencies of pDCs (CD19$^-$CD45RA$^+$CD11c$^+$), whereas FL$^{-/-}$ and DKO mice had approximately 6-fold reductions (Figure 3C-D; Table 1; supplemental Tables 1-2). In lymph nodes, cDCs were reduced by 3-fold in GM-CSF$^{-/-}$ and 28-fold in FL$^{-/-}$ mice, respectively (supplemental Figure 4; Table 1; supplemental Tables 1-2). However, there was no significan difference in the percentage of cDCs in DKO mice compared with either GM-CSF$^{-/-}$ or FL$^{-/-}$ mice, although DKO mice had significantl lower numbers of cDCs compared with GM-CSF$^{-/-}$ mice because of a total reduction in lymph node cellularity. FL$^{-/-}$ and DKO mice had similar reductions in the frequency of pDCs compared with WT or GM-CSF$^{-/-}$ mice; however, there was a significan reduction in absolute pDC numbers in DKO mice compared with FL$^{-/-}$ mice (supplemental Figure 4; Table 1; supplemental Tables 1-2). As in the spleen, FL$^{-/-}$ and DKO mice had similar frequencies of cDCs and pDCs in the bone marrow and liver (supplemental Figure 4).

Skin DCs are representative environmental interface tissue DCs that constitutively take up antigen and then migrate to draining lymph nodes where they represent a small fraction of DCs.[36] During inflammatio the migratory process is largely enhanced, and tissue-derived LN DCs increase in numbers. Analysis of epidermal sheets stained for MHCII showed an overall slight decrease of LCs in all knockout mice examined compared with WT mice with significan reductions of 2-fold, 1.3-fold, and 1.5-fold in GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice, respectively (Figure 4A; Table 1; supplemental Table 2). Relative numbers of dermal CD45$^+$MHCII$^+$ cells compared with total dermal CD45$^+$ cells were reduced in GM-CSF$^{-/-}$ and FL$^{-/-}$ mice compared with WT mice by 1.3-fold and 2.2-fold, respectively (Figure 4B-D; Table 1; supplemental Table 1). CD45$^+$MHCII$^+$ cells from DKO mice were even further reduced compared with FL or GM-CSF single cytokine knockout mice, with a 4.8-fold reduction compared with WT mice (Figure 4B,D; Table 1; supplemental Table 1). In the mouse, 3 distinct subsets of CD45$^+$MHCII$^+$ dermal-derived DCs have been characterized, based on a combination of surface protein expression. Langerhans cells (langerin$^+$CD11b$^+$) emigrating from the epidermis are found in the dermis, as well as the major dermis-resident DC population (langerin$^-$CD11b$^-$) and a recently identifie minor dermal DC subset, the langerin$^+$ dermal DCs (langerin$^+$CD11b$^-$).[37-39] Further subdivision of the dermal DC subsets into CD45$^+$MHCII$^+$ CD11b$^+$ or CD11b$^-$ cells showed that CD11b$^+$ dermal DCs were increasingly reduced from GM-CSF$^{-/-}$ to FL$^{-/-}$ to DKO mice compared with WT mice, with DKO mice having significan reductions compared with GM-CSF$^{-/-}$ and FL$^{-/-}$ mice (Figure 4C-D). In contrast, CD45$^+$MHCII$^+$CD11b$^-$ cells were predominately affected by the absence of FL, with comparable reduced frequencies in FL$^{-/-}$ and DKO mice (Figure 4C-D). Similar results were obtained when gating on CD45$^+$MHCII$^+$CD11b$^-$langerin$^+$ cells (data not shown).

Cells within the CD45$^+$MHCII$^+$ gate in the dermis include mostly DCs but also some MHCII^{lo} dermal macrophages. Nevertheless, the specifi reduction of dermal-derived DCs in mice lacking GM-CSF and FL was also evident when evaluating the CD40^{hi}CD11c^{int} constitutively migrating skin DCs in the draining LN, which include both migrated LCs and dermal DCs (Figure

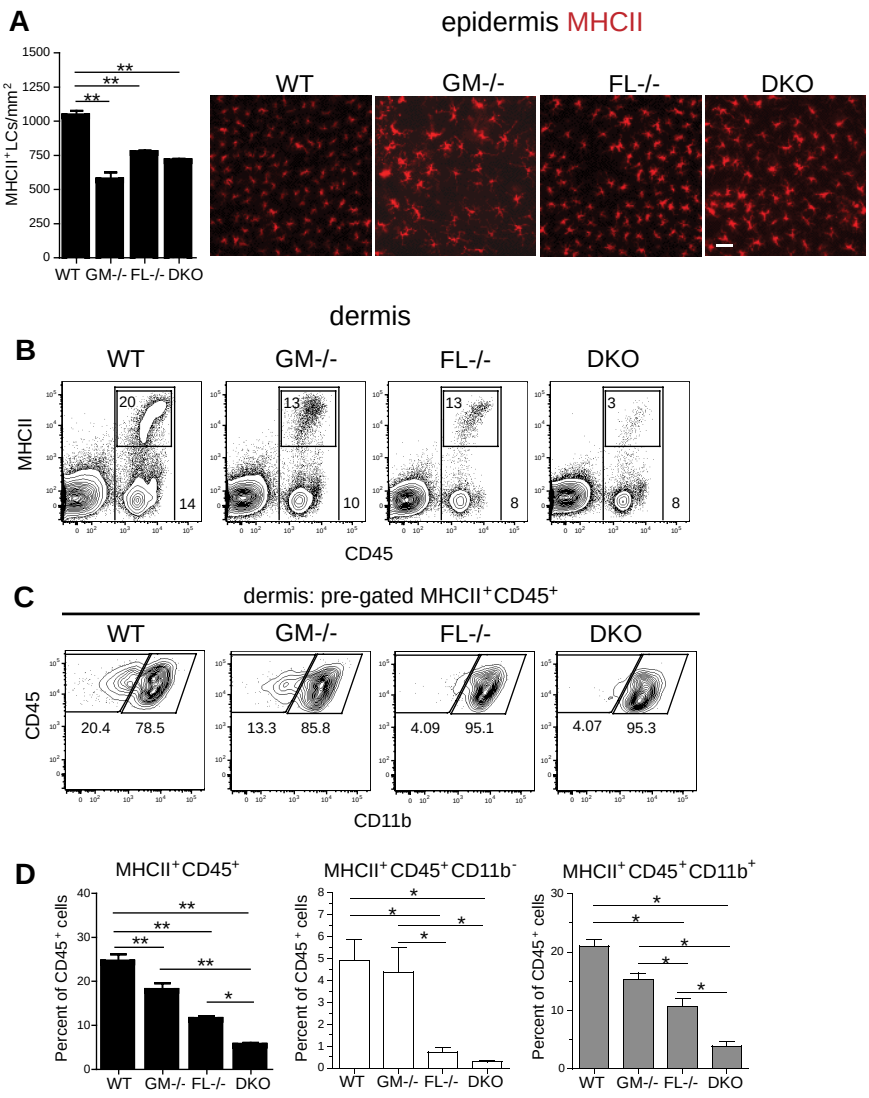

Figure 4. Dermal DCs are significantly reduced in the absence of GM-CSF and FL in steady state. DCs were analyzed from WT, GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice. (A) Immunofluorescence microscopy of MHCII-stained (red) epidermal sheets. MHCII$^+$ cells were counted on images taken from multiple fields per mouse (n = 4/group). Epidermal sheets were stained with PE-conjugated anti-MHCII antibody and mounted on slides with the use of Eukitt mounting medium. Images were taken at room temperature on a Nikon Eclipse E800 microscope with a CCD Qimaging camera using a Nikon Plan Apo 20×/0.75 NA objective lens and acquired with the use of OpenLab software. Scale bar represents 10 μm. (B-C) Flow cytometry of ex vivo–isolated dermal-derived cells. Representative FACS plots from WT, GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice are shown. (B) Percentage of all CD45$^+$ cells shown by outer gate. Inner gate represents percentage of MHCII$^+$ cells within CD45$^+$ gate. (C) Dermal DCs were pregated on MHCII$^+$CD45$^+$, followed by gating on CD11b$^+$ and CD11b$^-$ populations. (D) Percentages of total dermal-derived MHCII$^+$CD45$^+$ cells, as well as CD11b$^-$ and CD11b$^+$ subsets are shown. Results are given as the percentage of CD45$^+$ cells (n = 4-6 mice/group). *$P < .05$; **$P < .01$.

5A-B).[31] Importantly, only CD40^{hi}CD11c^{int} skin-derived DCs were significantl 2- and 3.5-fold lower in relative and absolute numbers, respectively, in DKO mice compared with FL$^{-/-}$ mice, whereas there were no substantial differences in the LN-resident CD40$^+$CD11c^{hi} or CD40$^-$CD11c^{int} DC subsets (Figure 5B; Table 1; supplemental Tables 1-2). Therefore, absence of either GM-CSF or FL caused significan reductions in the frequencies of dermal DCs and, unlike cDCs of the lymphoid organs, combined loss of

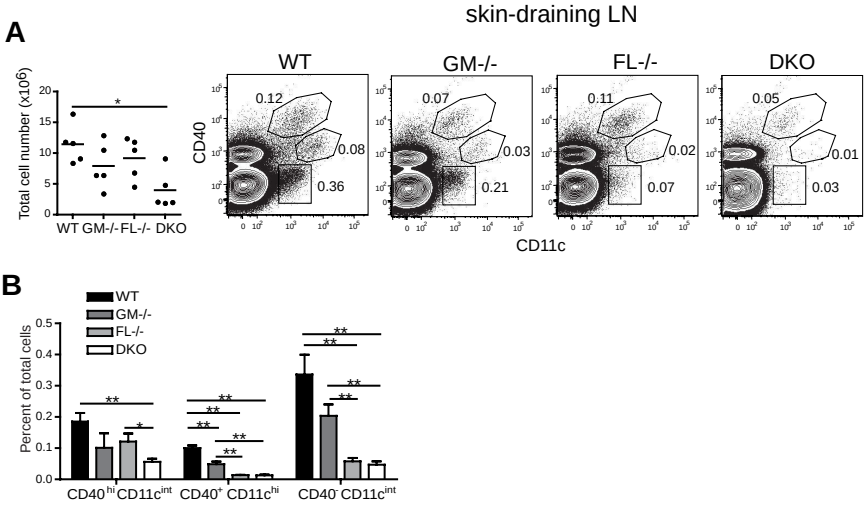

Figure 5. Skin-derived DCs in the draining lymph nodes are significantly reduced in GM-CSF– and FL-deficient mice in steady state. (A) Flow cytometry and analysis of DC subsets in skin-draining LNs (pooled inguinal, axillary, and cervical LNs). Horizontal lines indicate mean values of the results. LN DC subsets gated as CD40^{hi}CD11c^{int}, CD40$^+$CD11c^{hi}, and CD40$^-$CD11c^{int}. Total LN cell numbers (A) and percentage of each LN DC subset (B) are shown in graphs. *$P < .05$; **$P < .01$.

GM-CSF and FL further greatly diminished dermal DC numbers. Thus, both GM-CSF and FL are major cytokines involved in regulating dermal DC populations in the skin.

Reduced immune responses after subcutaneous immunization in the absence of GM-CSF and FL

Given that the strongest reduction in DCs in the absence of GM-CSF and FL was observed in steady-state dermal DCs at primary sites and in LNs, we examined the role of these cytokines in immune responses initiated at these sites. After contact sensitization with a FITC-containing solution of acetone and dibutylphthalate, the total number of FITC$^+$ CD40^{hi}CD11c^{int} cells in skin-draining LNs was consistently lower in DKO mice, compared with WT, GM-CSF$^{-/-}$, and FL$^{-/-}$ mice (supplemental Figure 5). DKO mice had significantl reduced total lymph node cellularity in steady state; however, by day 3 after FITC treatment, the LN cellularity of DKO mice was similar to FL$^{-/-}$ mice (supplemental Figure 5B). Still, the number of FITC$^+$ cells that migrated from the site of inflammatio to the draining LN was much lower in the DKO mice (supplemental Figure 5C). We speculate that the reduction of skin-derived cells observed in the LN after contact sensitization is most likely due to the reduction of dermal DCs in the skin. However, we cannot rule out an additional defect in maturation and migration of skin DCs, although CCR7 surface expression on CD40^{hi}CD11c^{int} cells from DKO mice was similarly up-regulated compared with WT mice (data not shown).

We next tested whether the reduction of dermal DCs in the draining LN in the absence of GM-CSF or FL or both would affect the activation and proliferation of CD4$^+$ T cells after immunization with whole protein antigen. To measure the proliferation of antigen-specifi T cells, mice were adoptively transferred with small numbers (6×10^4) of CFSE-labeled naive OVA-specifi CD4$^+$ OT-II T cells that homed at similar absolute numbers to skin-draining LNs of WT and knockout mice (data not shown). Mice were subsequently immunized subcutaneously with low amounts of OVA and monophosphoryl lipid A (MPL) as adjuvant. The proliferation of OVA-specifi OT-II T cells was assessed by flo cytometry. In GM-CSF$^{-/-}$ mice, OT-II T cells proliferated as well as in WT mice. FL$^{-/-}$ mice had a 1.4-fold reduction in the percentage of proliferating T cells compared with WT mice, whereas DKO mice had on average a 2-fold decrease in proliferating OT-II cells (Figure 6A). Similar results have also been shown by the lack of OT-I CD8$^+$ T-cell proliferation after immunization of CD11c$^+$ cell–depleted mice and by the dependence of proliferation and priming of CD4$^+$ DO11.10 T cells on the number of DCs migrating to the draining LN.[40,41] The decrease in T-cell activation in FL$^{-/-}$ and DKO mice was also most likely not due to a defect in maturation of the DCs, because they expressed MHCII and costimulatory molecules (CD40, CD80, and CD86) at levels similar to activated DCs from WT mice (data not shown). Thus, the reduction in OT-II cell proliferation after subcutaneous immunization can probably be attributed to the decrease in the number of antigen-capturing and migrating dermal DCs.

To assess whether a reduced immune response in GM-CSF and FL-deficien mice would also be observed in the absence of TCR-transgenic cells in a potentially more physiologic setting, T cell–dependent antibody responses were measured after subcutaneous immunization. During steady-state conditions no significan differences were seen in the basal immunoglobulin levels of IgM, IgA, and total IgG between WT, GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice (supplemental Figure 6). Mice were immunized subcutaneously with OVA and MPL, and OVA-specifi total IgG antibody levels were measured by ELISA. At early time points (less than 20 days after immunization), antibody levels were reduced in DKO mice and were significantl lower at day 7 compared with WT, GM-CSF$^{-/-}$, and FL$^{-/-}$ mice. After a booster immunization, both FL$^{-/-}$ and DKO mice had one-log lower antibody responses compared with WT or GM-CSF$^{-/-}$ mice (Figure 6B). Taken together, although GM-CSF, in combination with FL, plays an important role in the homeostasis of dermal DCs, as well as in the early events in the induction of adaptive immune responses, FL alone seems to be the more critical factor, compared with GM-CSF, contributing to the production of serum antibody levels after subcutaneous immunization.

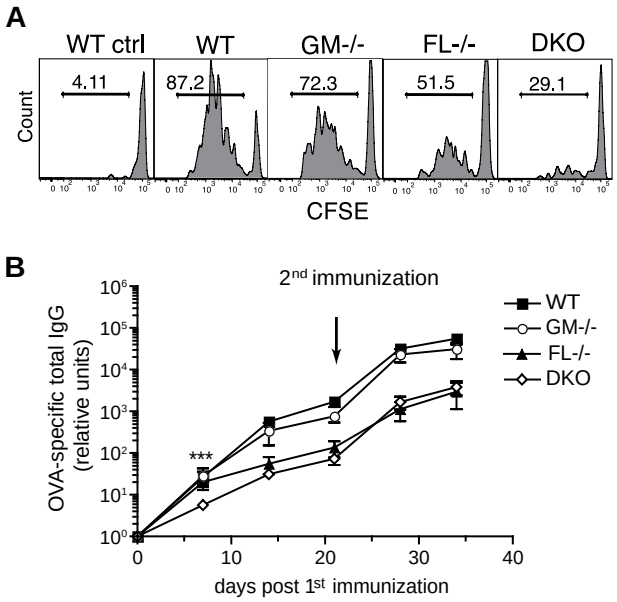

Figure 6. Reduced T-cell and antibody responses after subcutaneous immunization in the absence of GM-CSF and FL. (A) T-cell proliferation assay. Naive OT-II CD4$^+$CD45.1$^+$ T cells were sorted and labeled with CSFE before transfer into CD45.2$^+$ WT, GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice (6×10^4 cells/recipient). The next day, mice were immunized subcutaneously in the right flank with 2 μg OVA protein and 4 μg MPL. Draining LNs (right axillary and inguinal) and nondraining LNs (shown as representative WT control) were analyzed 3 days later. The proliferation of the transferred OT-II T cells was analyzed by gating on CD45.1$^+$CD3$^+$CD4$^+$ cells. Histograms show the percentage of OT-II T cells having diluted the CFSE label. Results are representative of 2 independent experiments with a total of 3 to 7 mice/group. (B) Production of OVA-specific IgG antibodies. WT, GM-CSF$^{-/-}$, FL$^{-/-}$, and DKO mice were immunized in the footpads with 2 μg whole OVA protein and 4 μg MPL, and serum was collected at the indicated time points. A second immunization was given at day 21. OVA-specific IgG antibodies were measured by ELISA (n = 7-8 mice/group). ***$P < .001$.

Discussion

GM-CSF and FL are 2 key cytokines for DC differentiation from progenitors. To date, the effects of GM-CSF and FL on DC development have been primarily studied in lymphoid organ cDC subsets in vivo or in vitro generated DCs. In this study we provide, for the firs time, a comprehensive analysis of in vivo DC *Gm-csfr* and *Flt3* receptor expression and the roles of GM-CSF and FL on the development of DCs from restricted progenitors in the BM to the various DC subsets throughout the body by studying GM-CSF$^{-/-}$, FL$^{-/-}$, and newly generated GM-CSF$^{-/-}$FL$^{-/-}$ double-deficien mice.

Although GM-CSF deficienc alone did not lead to a significan reduction of early DC progenitors such as MDP$^{\Delta}$s and CDPs in the

BM, FL deficienc , and even more so combined GM-CSF and FL deficienc led to a massive reduction of these populations. These results show that committed DC progenitors require GM-CSF and FL during development and/or maintenance in the BM, which is consistent with *Gm-csfr* and *Flt3* expression in both MDP$^{\Delta}$s and CDPs. This is in line with previous data showing CDP and MDP$^{\Delta}$ expansion upon in vivo stimulation with supraphysiologic levels of FL,[23,27] but it contrasts to previous finding in which no significan difference was seen in the numbers of MDP$^{\Delta}$s at 9 weeks of age in mice lacking the cognate receptor, Flt3.[23] We have obtained similar results in Flt3$^{-/-}$ mice as in the study by Waskow et al[23] (data not shown). These differential effects on MDP$^{\Delta}$ numbers between Flt3 receptor– and ligand-deficien mice will need further evaluation.

For steady-state cDCs in lymphoid organs, the current results confir previous finding in GM-CSF and FL single cytokine-deficien mice, with GM-CSF deficienc leading to minor, and FL deficienc leading to major reductions of cDCs.[10,15] Because GM-CSF was recently shown to inhibit FL-driven pDC development by STAT5-mediated IRF8 suppression, and increases in pDC frequencies are seen from STAT5-deficien progenitor cells,[6-8] it was critical to test if pDC numbers might be elevated in GM-CSF$^{-/-}$ mice. GM-CSF deficienc , however, had no effect on the number of pDCs in all the tissues analyzed. Thus, it is important to note that inhibitory signals from GM-CSF are not a primary mechanism in regulating pDC numbers in vivo, at least not under steady-state conditions.

Although FL is necessary to regulate steady-state numbers of lymphoid organ DCs, a small pool of DCs is still present in FL$^{-/-}$ mice. To determine whether these small numbers of DCs were maintained by a GM-CSF–driven pathway, we intercrossed GM-CSF$^{-/-}$ and FL$^{-/-}$ mice to generate double cytokine-deficien animals. Although double deficienc led to an additional reduction in BM DC progenitors as discussed earlier, it did not lead to further DC reduction compared with FL single deficienc in BM, spleen, liver, and LNs, thus showing compensation of the progenitor deficienc in double-deficien animals on the mature steady-state lymphoid organ DC level. However, analysis of nonlymphoid tissue DCs, ie, dermal DCs, showed significan reductions in both GM-CSF$^{-/-}$ and FL$^{-/-}$ mice with an additional substantial reduction in the combined knockout mice. Furthermore, whereas CD11b^{+} dermal DCs were progressively reduced from GM-CSF$^{-/-}$ to FL$^{-/-}$ to DKO mice, CD11b^{-} dermal DCs were primarily dependent on FL. These results suggest that these distinct dermal DC populations have differential cytokine requirements. Further studies will be required to determine the roles of GM-CSF and FL in the development, homeostasis, and subsequent function of these dermal DC subsets.

The deficienc of dermal DCs was confirme on detailed analysis of skin-derived DCs in LNs, suggesting consecutively reduced steady-state migration from skin.[3,36] These reduced steady-state DC numbers and migration might translate into impaired immune responses. Indeed, although not formally proven to be directly DC related, we observed increasingly reduced proliferative T-cell and specifi antibody responses from single- to double-deficien mice upon subcutaneous immunization. We thus identifie a synergistic role for both GM-CSF and FL during the differentiation or maintenance or both of DC progenitors and nonlymphoid organ dermal DCs during steady-state conditions.

Interestingly, the ex vivo analysis of *Gm-csfr* and *Flt3* expression on different lymphoid and nonlymphoid organ DCs closely correlated with the observed reductions of DCs in respective single and double cytokine knockout mice. This indicates that receptor expression not only is relevant for further differentiation or activation of these cells but also plays a role in their respective steady-state generation and maintenance. Because similar results for *Flt3* mRNA expression have also been shown for human DCs and myelomonocytic cells, we speculate that the differential and combined roles of these cytokines also hold true in humans.[42]

FL and GM-CSF are produced by stromal cells and activated T cells.[43,44] Although FL is constitutively expressed and measurable in serum, GM-CSF only becomes detectable in systemic inflammatio and then might drive a robust GM-CSF–induced pathway of DC differentiation involving monocytes.[3,11,12,43] It has, therefore, been suggested that the DC developmental pathways mediated by FL or GM-CSF are isolated events with FL contributing to DC development in steady state, whereas GM-CSF only plays a role in the differentiation of DCs from monocytes under inflammator conditions. However, our data show that small amounts of local GM-CSF expression must be involved in steady-state DC homeostasis, acting on both DC progenitors in BM and on DCs in nonlymphoid tissues. Furthermore, although FL and GM-CSF are major cytokines for DC development in steady state, cytokines such as IL-4, TNF-α, LTβ, M-CSF, and TGF-β1 will probably have more subtle effects or are only active in inflamma tory conditions.[3] Thus, future analysis of these cytokines in define tissues and the visualization of respective receptor-expressing cells should help to further defin DC differentiation pathways during steady state and inflammation

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Authorship

Contribution: D.K. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript; M.A.S., N.O., A.O.-O., and D.B. performed experiments and collected data; and M.G.M. directed the study and cowrote the manuscript.

Conflict-of-interes disclosure: The authors declare no competing financia interests.

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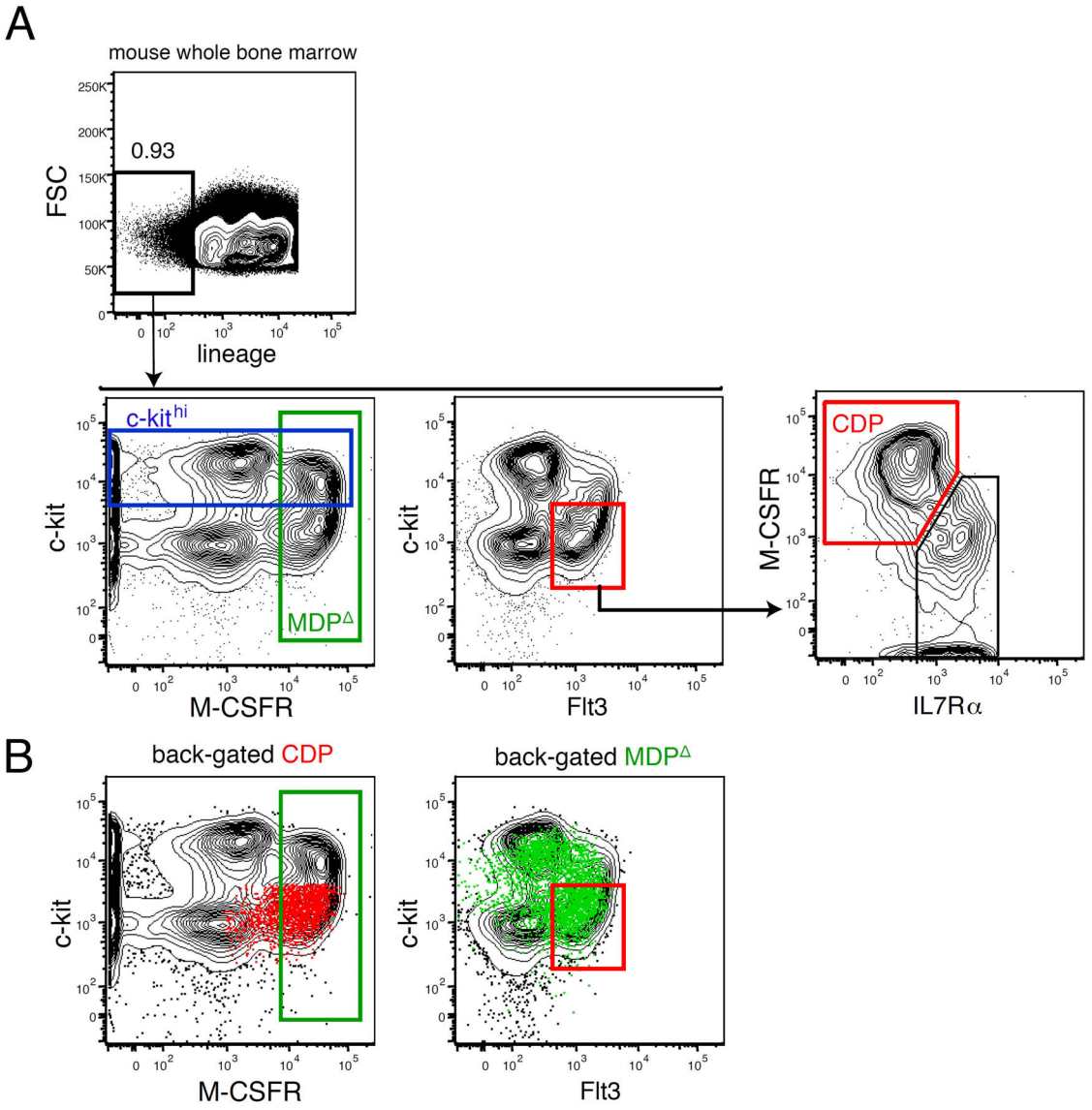
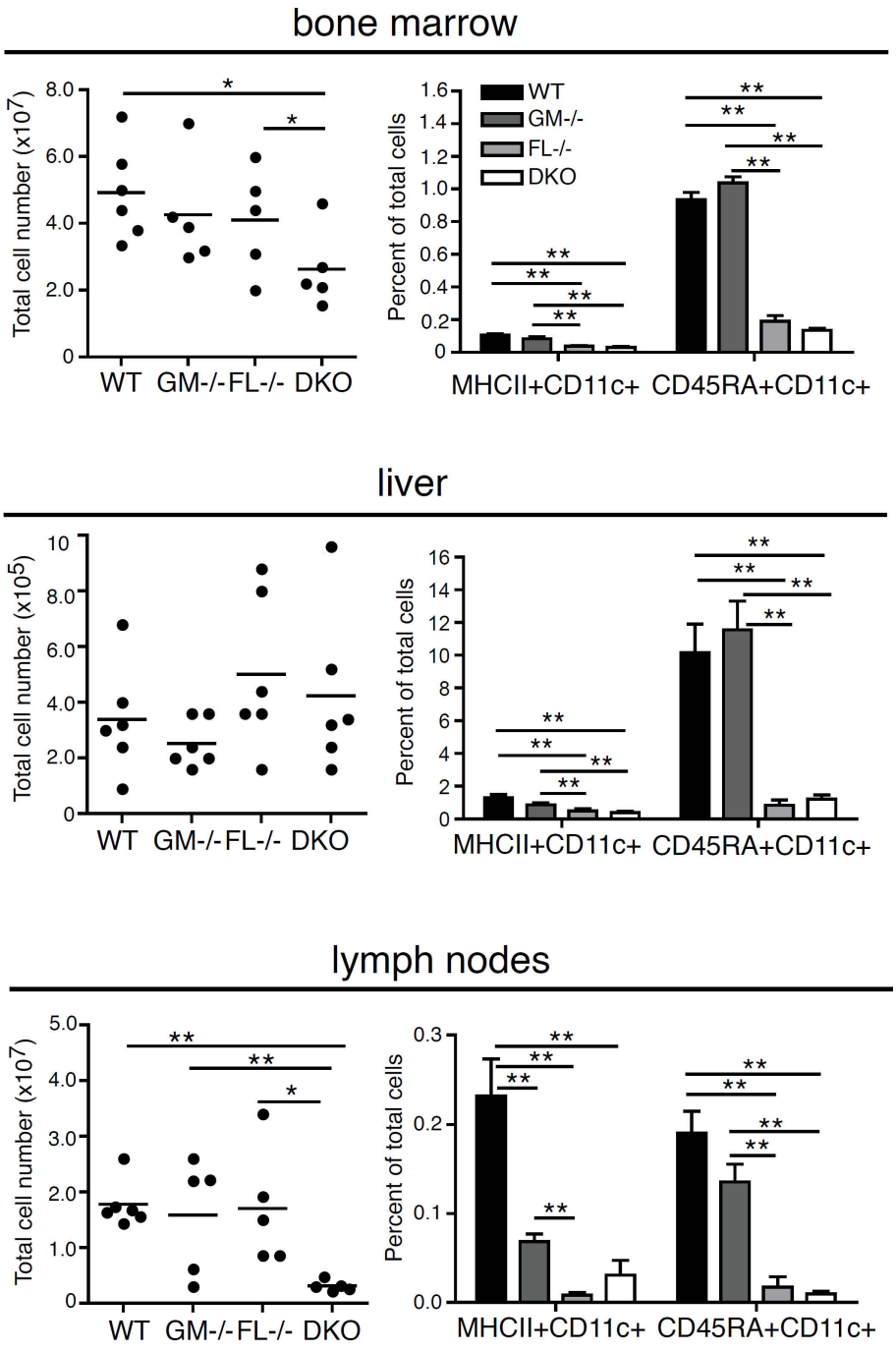
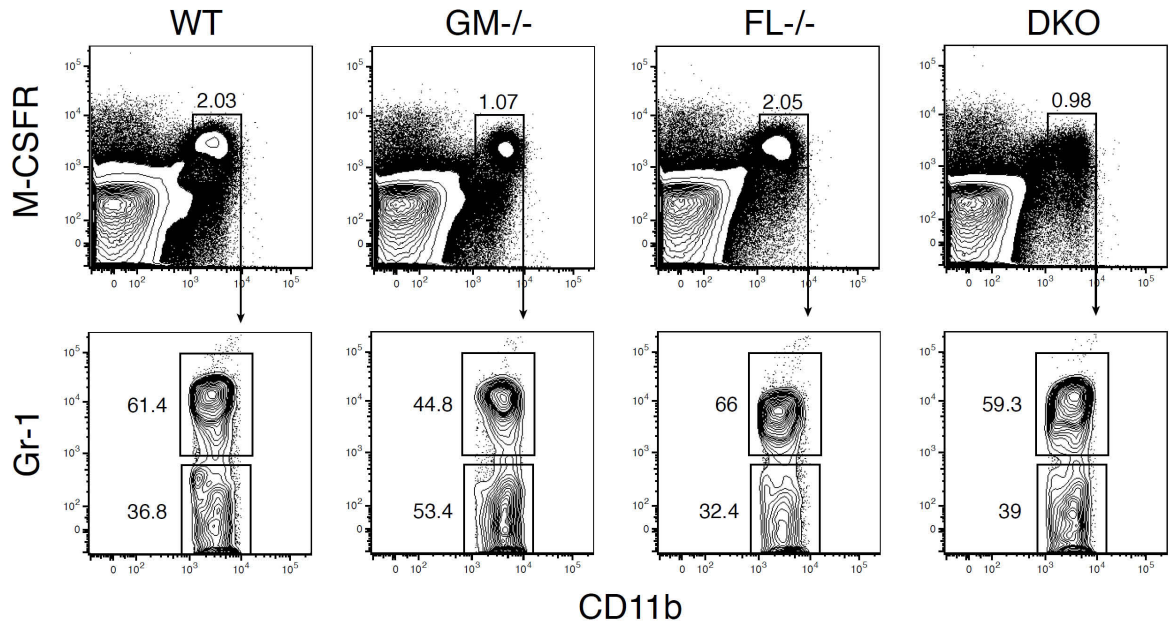


Supplementary Figure S1. Population comparisons between MDP, and CDP.

(A) Gating strategy of each population from lineage negative cells. c-kit^{hi} (blue), MDP (green), and CDP (red). (B) Comparative back-gating of MDP and CDP progenitor populations on lin- c-kit vs. Flt3 and lin- c-kit vs. M-CSFR.



Supplementary Figure 2. Genotypic analysis and cytokine serum levels in GM-CSF/FL-/- DKO mice. (A) Representative genotyping of DKO mice generated by crossing GM-CSF-/- and FL-/- mice. PCR analysis of deleted gene sequences of Flt3l and Gm-csf, and -globin, as a positive control. (B) Steady-state serum levels of FL, GM-CSF, and M-CSF (pg/ml) in WT, GM-CSF-/-, FL-/-, and DKO mice. n.d., not detectable.



Supplementary Figure 3. Gating strategy for monocyte subsets in DKO mice.

Monocytes from the spleen were first gated as M-CSFR^{+} CD11b^{+} and then divided into Gr-1^{+} and Gr-1^{-} subsets. Representative FACS plots are shown from WT, GM-CSF-/-, FL-/- and DKO mice.

Chapter 13

Isolation of Common Dendritic Cell Progenitors (CDP) from Mouse Bone Marrow

Nobuyuki Onai, Markus G. Manz, and Michael A. Schmid

Abstract

In the steady-state lymphoid organ, dendritic cells (DCs) are classified into two major subsets, plasmacytoid DC (pDC) and conventional DC (cDC). A standing question was whether a common progenitor for plasmacytoid and conventional dendritic cells exists during the sequential differentiation from hematopoietic stem cells to dendritic cells. We have recently identified such a common clonogenic plasmacytoid and dendritic cell progenitor (CDP) from mouse bone marrow using antibodies for c-kit, Flt3, and M-CSFR. CDPs generated almost exclusively pDC and cDC in vitro and upon transfer in irradiated and steady-state mice in vivo. Single-cell analysis revealed the existence of clonal progenitors giving rise to both pDC and cDC within the CDP population. Thus, these results prove the existence of a common developmental pathway for at least some pDCs and cDCs in lymphoid organs in vivo.

Key words: Flt3, M-CSFR, hematopoietic progenitor, dendritic cell (DC), plasmacytoid DC (pDC), conventional DC (cDC).

1. Introduction

Dendritic cells (DCs) possess strong antigen-presenting ability and are spread throughout the body (1). In the peripheral tissue, DCs capture the antigen, migrate to the draining lymph nodes, and present it to T cells to initiate adaptive immunity (2). DCs also act as an important regulator for tolerance in the steady state (3). In the secondary lymphoid tissue, resident DCs comprise a heterogeneous group and can be classified into at least two groups, plasmacytoid DC (pDC) and conventional DC (cDC). cDCs are further subdivided into three groups such as

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$CD11c^+CD8\alpha^-CD4^-CD11b^+$, $CD11c^+CD8\alpha^-CD4^+CD11b^+$, and $CD11c^+CD8\alpha^+CD4^-CD11b^-$ dendritic cells. cDCs efficiently activate naïve T cells by presentation of antigen on MHC class II. $CD8\alpha^+$ DCs, however, have a high potential to uptake and cross-present exogenous antigen via MHC class I. pDCs produce high amounts of type-I interferon upon stimulation by viruses and CpG and are consequently as well called type-I interferon-producing cells (IPCs). Thus, pDCs are crucial regulator of antiviral immunity (4).

All DC subsets continuously differentiate from hematopoietic stem cells via intermediate committed progenitors (5, 6). However, it was not clear so far, whether DC subsets derive from separate progenitors or one common progenitor. Based on shared cytokine dependencies, the analysis of gene-deficient mice and the biology of plasmacytoid and conventional dendritic cells, it has been suggested that these cells might proceed through DC-restricted common developmental intermediates.

Indeed, Flt3–L ligand is the only cytokine to induce differentiation of both pDC and cDC from mouse bone marrow cells (7). Flt3L-deficient mice show only about one-tenth the numbers of lymphoid tissue pDCs and cDCs compared to wild-type mice (8). Furthermore, either STAT3- or PU.1-deficient mice, which are down-stream transcription factors for Flt3-signaling, showed severe reduction of both pDC and cDC numbers (6, 9, 10). These results suggest that a common DC progenitor should exist and it might express the cytokine receptor Flt3.

Recently, we identified and characterized lin^- c-$kit^{int}Flt3^+$M-$CSFR^+$ cells in mouse bone marrow that on a clonal level in vitro, and as a population in vitro and in vivo, efficiently generate bone marrow, spleen, and lymph node pDCs and cDCs, but no other lineage read out (11, 12). Thus, we call these cells common dendritic cell progenitors (CDP) (11). These cells thus define a common Flt3 responsive pathway for steady-state DC maintenance.

2. Materials

2.1. Preparation of Bone Marrow Cell Suspension

1. C57BL/6 mice, 8–12 weeks old.
2. 70% Ethanol.
3. Phosphate-buffered saline (PBS).
4. 10-ml Syringes with 21-ga needles.
5. Mortar and pestle.
6. Nylon meshes (70 μm pore size).
7. Histopaque-1077 (Sigma–Aldrich).

2.2. Pre-enrichment of the Lineage Negative Fraction of Bone Marrow Cells

1. PE-Cy5-conjugated antibodies against lineage antigens (CD3ϵ, 145-2C11; CD4, GK1.5; CD8α, 53-6.7; B220, RA3-6B2; CD19, MB19-1; CD11b, M1/70; Gr-1, RB6-8C5; TER119, TER119; NK1.1, PK136).
2. Staining buffer: 2% fetal calf serum (FCS), 2 mM EDTA in PBS.
3. Anti-Cy5/Anti-Alexa Flour 647 microbeads (Miltenyi Biotec).
4. LS MACS columns and MidiMACS Separator or AutoMACS (Miltenyi Biotec).

2.3. Antibody Staining and Cell Sorting

1. Staining buffer: 1% FCS, 2 mM EDTA in PBS stored at 4°C.
2. Primary antibodies: FITC-conjugated anti-CD127 (A7R34), PE-conjugated anti-CD135 (A2F10.1), APC-conjugated anti-c-kit (ACK2), and biotin-conjugated anti-CD115 (AFS-98) (eBioscience).
3. Second antibody: streptavidin-APC-Cy7 (eBioscience).
4. Propidium iodide solution (1,000X) (Sigma) is dissolved at 10 mg/ml in PBS and stored at 4°C in the dark (light sensitive).
5. Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin.
6. Cell sorter: BD FACSAria (Becton Dickinson Immunocytometry Systems) or MoFlo (Beckman Coulter).

2.4. In Vitro Myeloid, Erythroid, and Lymphoid Differentiation Culture

1. Methylcellulose medium: MethoCult M3231 (StemCell Technologies).
2. Recombinant cytokines: mSCF (R&D), mIL-3 (R&D), mIL-11(R&D), human Flt3-ligand (R&D), mGM-CSF (R&D), mTpo (R&D), hEpo (Roche), mM-CSF (R&D), mIL-7 (R&D).
3. 5-ml Syringes with 18-ga needles.

2.5. In Vitro DC Differentiation Culture

1. IMDM supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin.
2. Recombinant human Flt3-ligand (R&D).
3. Staining buffer: 1% FCS, 2 mM EDTA in PBS stored at 4°C.
4. Staining antibodies: FITC-conjugated anti-I-A/I-E (M5/114.15.2) (eBioscience), PE-conjugated anti-PDCA-1 (Miltenyi Biotec), PE-conjugated anti-CD45RA (14.8) (BD Bioscience), APC-conjugated anti-CD11c (N418) (eBioscience).

5. Propidium iodide solution (1,000X) (Sigma) is dissolved at 10 mg/ml in PBS and stored at 4°C in the dark (light sensitive).

3. Method

3.1. Preparation of Bone Marrow Cell Suspension

1. Wet the whole body of the mouse with 70% ethanol for sterilization.
2. Remove femurs, tibias, and the backbone from five mice and place them into ice-cold PBS. Remove the muscles from the bones using scissors and forceps and transfer them into a new Petri dish containing PBS.
3. Add 10 ml of ice-cold PBS into a mortar and crash/grind the bones using a pestle, or add 10 ml of ice-cold PBS into dish, and flushing out marrow using syringe with needle to obtain a bone marrow cell suspension from bone shaft. Pass the cell suspension through a nylon mesh to remove debris.
4. Add 10 ml of ice-cold PBS into mortar and transfer cleaned backbone. Crash and grind the backbone using pestle to obtain spinal marrow. Remove and discard the white funiculus that as well will be extracted during the crushing. Pass the cell suspension through the nylon mesh to remove debris. Mix bone marrow and spinal marrow cell suspensions and centrifuge for 5 min at room temperature.
5. During centrifugation, add 5 ml of room temperature Histopaque-1077 into a 15-ml tube.
6. Remove supernatant and resuspend cells in 5 ml of PBS at room temperature. Carefully overlay 5 ml of cell suspension onto Histopaque-1077.
7. Centrifuge for 30 min at 18–20°C, 900 × *g* with acceleration and brakes set to "zero."
8. After centrifugation, carefully aspirate the uppermost layer. Subsequently, transfer the intermediate mononuclear cell layer into a new tube. Wash the cells with an excess of ice-cold PBS (5–10 volume) and centrifuge for 5 min at 4°C.
9. Cells are resuspended in PBS and counted.

3.2. Pre-enrich the Lineage Negative Cell Fraction

1. Centrifuge cell suspension at 400 × *g* for 5 min at 4°C and aspirate supernatant.
2. Add PE-Cy5-conjugated antibody cocktail against lineage antigens (CD3, CD4, CD8, B220, CD19, CD11b, Gr-1, TER119, and NK1.1) to the cells, mix well, and incubate for 30 min at 4°C in the dark.

3. Wash the cells with ice-cold staining buffer in excess (5–10 × volume), centrifuge for 5 min at 4°C, and aspirate the supernatant.
4. Resuspend the cells in staining buffer, add appropriate volume of anti-Cy5/Anti-Alexa Flour 647 microbeads according to manufacturer's instructions, and incubate for 15 min at 4°C in the dark.
5. Wash the cells with ice-cold staining buffer in excess, centrifuge for 5 min at 4°C, and aspirate supernatant.
6. After resuspending the cells in staining buffer, proceed with magnetic separation to obtain the lineage-negative cell fraction using MidiMACS Separator or AutoMACS according to manufacturer's instructions.

3.3. Antibody Staining and Cell Sorting

1. Centrifuge lineage negative cell suspension at 400 × *g* for 5 min at 4°C and aspirate supernatant.
2. Add primary antibody mix to the cell suspension, mix well, and incubate for 30 min at 4°C in the dark.
3. Wash the cells with ice-cold staining buffer in excess, centrifuge for 5 min, and aspirate supernatant.
4. Add secondary antibody to the cells, mix well, and incubate for 30 min at 4°C in the dark.
5. Wash the cells with ice-cold staining buffer in excess, centrifuge for 5 min, and aspirate supernatant.
6. Cells are resuspended in staining buffer containing propidium iodide (final concentration 10 μg/ml) to stain and exclude dead cells.
7. Sort the lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα$^-$ cell fraction by using a cell sorter, as shown in **Fig.13.1 A.** Make sure to include as well M-CSFR low positive cells.
8. Target cells are sorted into a tube containing 1 ml of 10% FCS–IMDM medium.
9. Freshly isolated lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα$^-$ cells do not express DC-related cell surface marker such as CD11c, MHC class II, PDCA-1, and CD40, as shown in **Fig. 13.1 B.**

3.4. In Vitro Myeloid, Erythroid, and Lymphoid Differentiation Assay

1. Add sorted lin$^-$c-kit^{high} cells, lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7R$^-$ cells (R1), or lin$^-$c-kit^{int}Flt3$^+$IL-7R$^+$ (R2) cells to 3 ml of methylcellulose medium, MethoCult M3231, and add cytokine cocktail for myeloid erythroid colony-forming assay (10 ng/ml mSCF (R&D), 10 ng/ml mIL-3 (R&D), 10 ng/ml mIL-11(R&D), 10 ng/ml human Flt3-ligand (R&D), 10 ng/ml mGM-CSF (R&D), 10 ng/ml mTpo

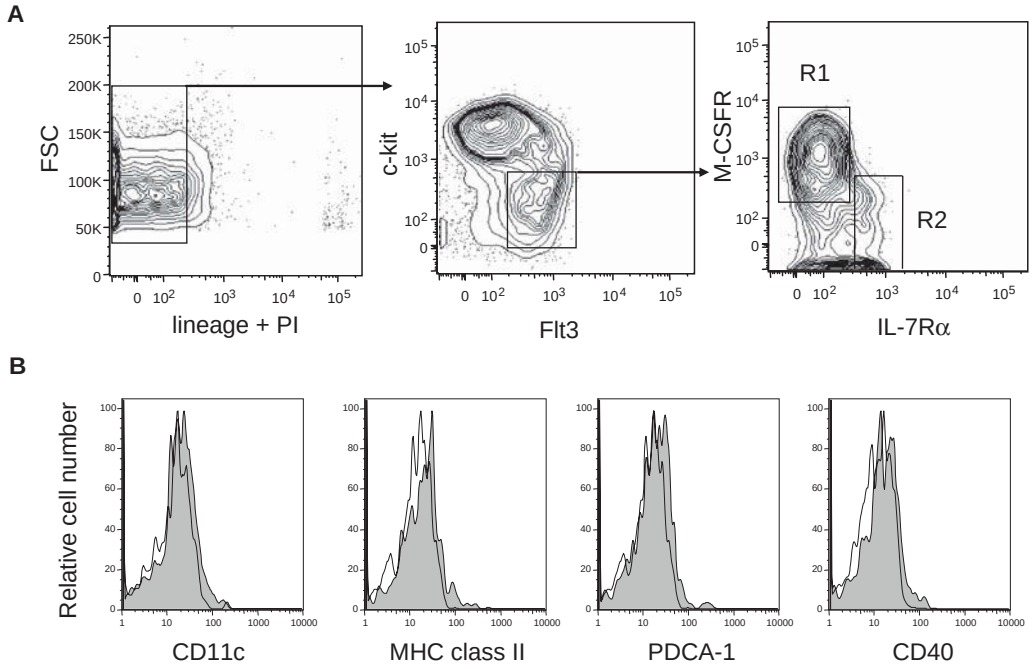

Fig. 13.1. Identification of lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ progenitor cells in mouse bone marrow. (**A**) lin$^-$ cells were divided by c-kit and Flt3 expression (*middle panel*). lin$^-$c-kit^{int}Flt3$^+$ cells contained Flt3$^+$M-CSFR$^+$ (R1) and Flt3$^+$IL-7Rα^+ (R2) cells (*right panel*). (**B**) Additional surface marker expression on lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ cells (closed histograms) and respective isotype controls (open histograms). Freshly isolated lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ cells from bone marrow do not express the DC-related markers CD11c, MHC class II, PDCA-1, and CD40.

(R&D), 1 U/ml hEpo (Roche)), for macrophage colony forming assay; 10 ng/ml M-CSF, for pre-B cell colony-forming assay; 10 ng/ml SCF, 10 ng/ml mIL-7, 10 ng/ml human Flt3-ligand.

2. Mix vigorously because methylcellulose medium has a high viscosity.
3. Leave the mixture to stand for 10 min at room temperature until air bubbles have disappeared.
4. Take up the methylcellulose medium by a 5-ml syringe with a 18-ga needle, and pour them into a cell culture dish.
5. Determine and enumerate colonies under an inverted microscope consecutively from day 3 to day 8.
6. To confirm colony-types, pick colonies using a fine-drawn Pasteur pipette, spin them on slides, Giemsa stain them and evaluate by light microscopy.
7. Lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα^- cells completely lacked CFU-GEMM, CFU-G, CFU-MegE, CHU-Meg, BFU-E, and CFU-B as shown in **Fig. 13.2A** and **C.** Less than 4% of

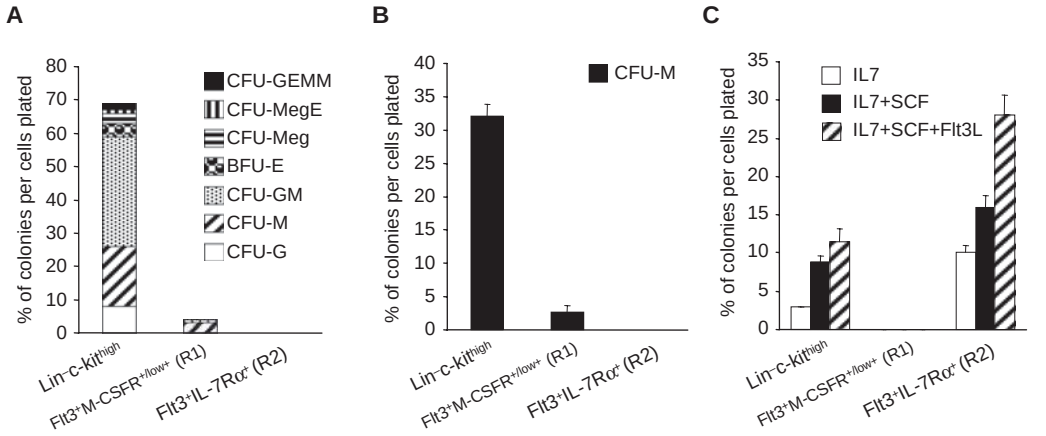

Fig. 13.2. In vitro colony-forming activity of lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ progenitor cells. (**A**) Myeloid, (**B**) macrophage, (**C**) and pre-B cell colony-forming unit activity of lin$^-$c-kit^{high} cells, lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ cells (R1), and lin$^-$c-kit^{int}Flt3$^+$IL-7Rα^+ (R2) cells (as indicated in **Fig.13.1A**); 200 cells were plated each. Colony-forming unit (CFU) activity. GEMM, granulocyte–erythrocyte–macrophage–megakaryocyte; MegE, megakaryocyte–erythrocyte; Meg, megakaryocyte; BFU-E, burst-forming unit, erythrocyte; GM, granulocyte-macrophage; M, macrophage; G, granulocyte; B, B cell.

lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα^- cells gave rise to CFU-GM and CFU-M as shown in **Fig. 13.2 A** and **B**.

3.5. In Vitro Culture and FCM Analysis

1. Culture the sorted lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα^- cells in IMDM supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml Flt3-ligand (R&D).
2. Replace half of the medium every 3 days with medium containing twofold cytokines added.
3. After 8 days of culture, harvest offspring cells, transfer into a tube, centrifuge at 400 × *g* for 5 min at 4°C, and aspirate supernatant.
4. Add appropriate amount of monoclonal antibody mixture to the cell pellet and mix well.
5. Incubate for 30 min at 4°C in the dark.
6. Add 500 μl of buffer; centrifuge at 250 × *g* for 5 min.
7. Resuspend samples in 1 ml of buffer containing propidium iodide (final concentration 10 μg/ml) to stain and exclude dead cells and keep at 4°C.
8. Cells are analyzed using a FACSCalibur and a FACSCanto (Becton Dickinson Immunocytometry Systems) according to manufacturer's instructions.
9. The lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$IL-7Rα^- cells gave rise to both CD11c$^+$B220$^+$CD45RA$^+$PDCA-1$^+$ pDC and CD11c$^+$B220$^-$CD45RA$^-$PDCA-1$^-$ conventional DCs in this culture condition, as shown in **Fig.13.3.**

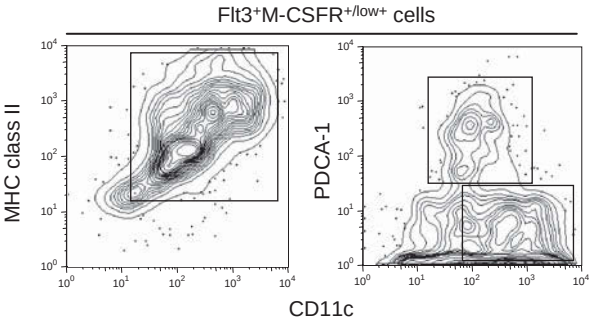

Fig. 13.3. lin^-c-$kit^{int}Flt3^+M\text{-}CSFR^+$ progenitors generate pDCs and cDCs in vitro. Lin^-c-$kit^{int}Flt3^+M\text{-}CSFR^+$ cells were cultured with recombinant human Flt3-ligand supplemented media for 8 days.

4. Notes

It is important to perform all procedures under sterilize conditions.

For the sorting, it is important to set up compensation tubes of stains in single colors for multi-color analysis.

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nature immunology

Identification of clonogenic common Flt3$^+$M-CSFR$^+$ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow

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Lymphoid tissue plasmacytoid and conventional dendritic cells (DCs) are continuously regenerated from hematopoietic stem cells. The cytokine dependence and biology of plasmacytoid and conventional DCs suggest that regeneration might proceed through common DC-restricted developmental intermediates. By selecting for cytokine receptor expression relevant to DC development, we identify here highly cycling Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$ cells with a distinct gene-expression profile in mouse bone marrow that, on a clonal level *in vitro* and as a population both *in vitro* and *in vivo*, efficiently generated plasmacytoid and conventional DCs but no other lineages, which increased in number after *in vivo* injection of the cytokine Flt3 ligand. These clonogenic common DC progenitors thus define a cytokine-regulated DC developmental pathway that ensures the supply of various DC populations.

Dendritic cells (DCs) are critical for the initiation of immune responses and for the maintenance of self-tolerance[1,2]. On the basis of their location, phenotype and function, many DC subsets have been identified in mice and men[3]. Mouse spleen and lymph node DCs consist at least of two main populations: CD11c$^+$B220$^+$ CD45RA$^+$PDCA-1$^+$ plasmacytoid DCs (pDCs), also called 'natural type I interferon–producing cells' or 'plasmacytoid pre-DCs'; and CD11c$^+$B220$^-$CD45RA$^-$PDCA-1$^-$ conventional DCs (cDCs), which are further categorized as CD11c$^+$CD8α$^-$CD4$^-$CD11b$^+$, CD11c$^+$ CD8α$^-$CD4$^+$CD11b$^+$ and CD11c$^+$CD8α$^+$CD4$^-$CD11b$^-$ subpopulations[3]. In steady-state lymphoid organs, 0.3–5% of DCs are dividing and their half-life is 1.5–7 d (refs. 3–6). Although few DCs in lymphoid organs might divide over prolonged time *in situ*[5], data on the separation of parabiotic mice have confirmed previous conclusions from non–self-renewing progenitor cell–transfer experiments: most spleen and lymph node DCs must be continuously replaced through blood or lymph, probably by cells originating from hematopoietic stem and progenitor cells in the bone marrow[6–9].

Hematopoietic differentiation is a regulated, continuous, multilinear process in which hematopoietic stem cells (HSCs) give rise to cells with progressively limited developmental options[10]. On the basis of cell surface phenotypes and response to environmental stimuli, several early developmental intermediates have been defined in mice and men[9,11–13]. In contrast to initial expectations, some lymphoid and myeloid committed progenitors maintain both pDC and cDC developmental capacity[7,8,14,15], a property subsequently found to be confined to the Flt3$^+$ fractions among these progenitor cells[16–18].

Flt3 is a receptor tyrosine kinase with homology to the kinases c-Kit and c-Fms[19]. Flt3 ligand (Flt3L) is sufficient to induce the differentiation of both pDCs and cDCs from progenitors *in vitro*[20], and it supports the maintenance of DCs in steady-state lymphoid organs *in vivo*: Flt3L-deficient mice, mice with hematopoietic deletion of the gene encoding STAT3 (a transcription factor relevant in signaling 'downstream' of Flt3) and mice treated with Flt3 kinase inhibitors have about 10% the pDCs and cDCs of untreated mice[21–23]. Conversely, injection or expression of Flt3L in mice increases pDCs and cDCs, with up to 30% of splenocytes expressing CD11c[17,24,25], and enforced expression of Flt3 in Flt3$^-$ and Flt3$^+$ bone marrow progenitors restores and enhances their *in vitro* and *in vivo* development into pDCs and cDCs[26]. Furthermore, all steady-state spleen DC subtypes but no other mature hematopoietic cell populations express Flt3 (ref. 17). Thus, Flt3-Flt3L regulates the development and maintenance of lymphoid organ DCs in a nonredundant way.

Although granulocyte-macrophage colony-stimulating factor (GM-CSF) is effective in generating DCs from both mouse and human monocytes and hematopoietic progenitors *in vitro*[27–29], mice lacking GM-CSF or its receptor have normal or only slightly lower steady-state lymphoid tissue DC numbers[30] (unpublished data). M-CSF is effective in the differentiation of macrophages but not DCs *in vitro*[31], and mice lacking M-CSF or its receptor (M-CSFR) have relatively normal numbers of steady-state lymphoid tissue DCs, whereas monocytes

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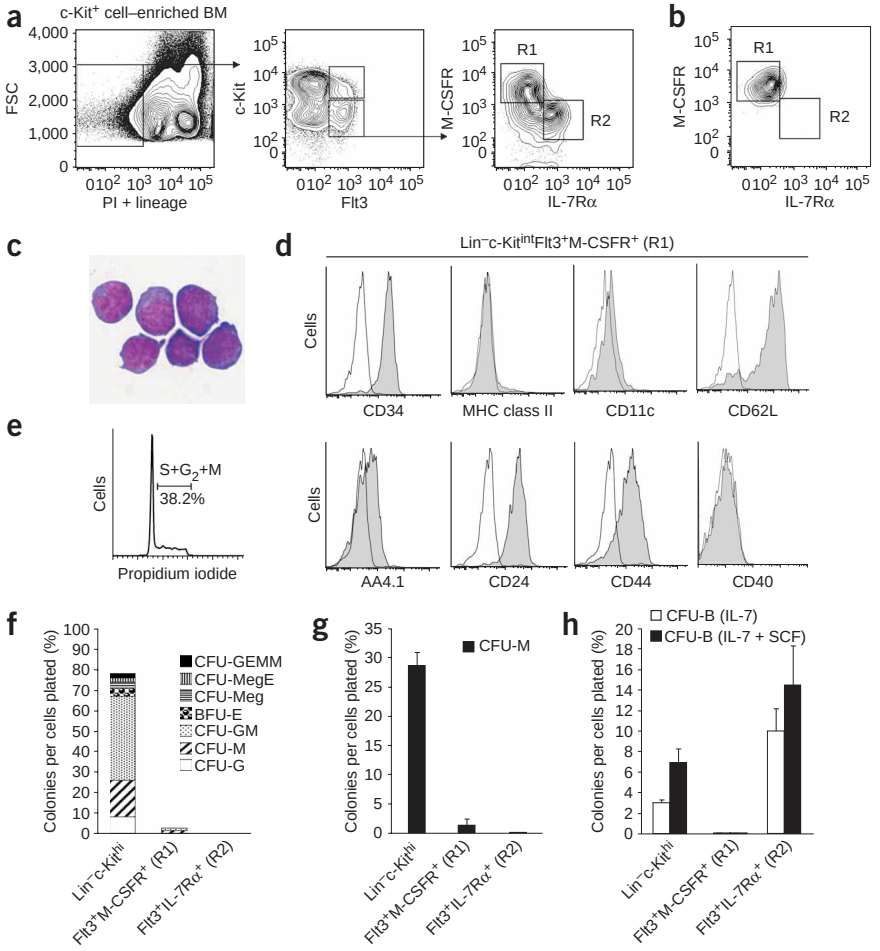

Figure 1 Identification of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ progenitor cells in mouse bone marrow. (**a**,**b**) Flow cytometry of Lin⁻ cells for the expression of c-Kit and Flt3 (**a**, middle) and to identify Flt3⁺M-CSFR⁺ cells (R1) and Flt3⁺IL-7Rα⁺ cells (R2). (**a**) Middle, top gate, Lin⁻c-Kit^{hi}Flt3⁺; middle, bottom gate, Lin⁻c-Kit^{int}Flt3⁺; right, R1, Lin⁻c-Kit^{int}Flt3⁺IL-7Rα⁻M-CSFR⁺; right, R2, Lin⁻c-Kit^{int}Flt3⁺IL-7Rα⁺M-CSFR⁻. BM, bone marrow; FSC, forward scatter; PI, propidium iodide. (**b**) Double-sorted Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ (R1) population. (**c**) May-Grünwald-Giemsa staining of a cytospin of sorted Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells. Original magnification, ×1,000. (**d**) Flow cytometry of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells for the expression of surface markers (shaded) and the respective isotype controls (open). (**e**) Analysis of the cell cycle status of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells by propidium iodide staining. (**f**–**h**) *In vitro* assays of Lin⁻c-Kit^{hi} cells, Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ (R1) cells and Lin⁻c-Kit^{int}Flt3⁺IL-7Rα⁺ (R2) cells (as in **a**) for myeloid (**f**), macrophage (**g**) and pre–B cell (**h**) colony-forming unit (CFU) activity. For each sample represented by a bar, 200 cells were plated. GEMM, granulocyte-erythrocyte-macrophage-megakaryocyte; MegE, megakaryocyte-erythrocyte; Meg, megakaryocyte; BFU-E, burst-forming unit, erythrocyte; GM, granulocyte-macrophage; M, macrophage; G, granulocyte; B, B cell; SCF, stem cell factor. Data are representative of one experiment of more than twenty (**a**,**b**), three (**c**,**d**), two (**e**) or five (**f**–**h**).

and epidermal Langerhans cells do not develop in these mice[32,33]. Notably, however, M-CSFR is expressed by both pDCs and cDCs[34], and M-CSFR⁺ human bone marrow progenitor cells have pDC differentiation ability[35]. Thus, GM-CSF and M-CSF are probably important in the development and activation of nonlymphoid tissue DCs and inflammatory DCs but seem dispensable for the maintenance of lymphoid organ DCs.

Several immediate DC progenitor populations have been identified in mice. CD11c⁺CD31⁺Ly6C⁺ cells in bone marrow and blood give rise to macrophages, cDCs and pDCs *in vitro*[36]. CD11c⁺MHCII⁻ peripheral blood cells generate cDCs and pDCs but no other cells *in vivo*[37]. Lin⁻c-Kit⁺CX3CR1⁺ bone marrow cells isolated from *Cx3cr1-gfp* mice differentiate exclusively into macrophages and cDCs on a clonal level *in vitro* and as a population *in vivo*[38]. CD11c^{int}CD45RA^{lo}CD43^{int}SIRP-α^{int}MHCII⁻ spleen cells generate all cDCs but no other cells *in vitro* and *in vivo*[39]. Although such studies have enhanced the understanding of DC development, they have not addressed the issue of whether, during sequential DC differentiation, single cells with combined pDC- and cDC-restricted differentiation ability exist that contribute to steady-state DC development.

Here we excluded defined progenitor cell populations and focused on DC development relevant cytokine receptor expression. We report the characterization of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells in mouse bone marrow that, on a clonal level *in vitro* and as a population *in vitro* and *in vivo*, efficiently generated bone marrow, spleen and lymph node pDCs and cDCs but no other cell lineages. These Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells thus define a common Flt3-responsive pathway for steady-state DC maintenance.

RESULTS

Identification of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ bone marrow cells

In mouse bone marrow, the mature lineage surface marker–negative (Lin⁻) fraction contains most if not all HSCs and early progenitor cells[10]. DC developmental potential is confined to Lin⁻Flt3⁺ cells[16,17,26]. However, as Flt3L also supports the development of other hematopoietic lineages, Flt3 expression is not exclusive but is also present in short-term HSCs, multipotent progenitor cells, and the main fractions of myeloid progenitor cells (common myeloid progenitors and granulocyte-macrophage progenitors) and lymphoid progenitor cells (common lymphoid progenitors) that all also have DC differentiation potential[16,17,26,40,41]. To identify putative common DC progenitors, we thus needed to exclude Lin⁻Flt3⁺ fractions with alternative developmental options and to explore additional cell surface expression of commitment-defining proteins. Of two candidate cytokine receptors, the GM-CSF receptor and M-CSFR, only antibodies to M-CSFR were available to us.

The Lin⁻c-Kit^{hi}Flt3⁺ cell population (**Fig. 1a**) includes at least some short-term HSCs, multipotent progenitors, common myeloid progenitors and granulocyte-macrophage progenitors, and indeed we were unable to identify a population that lacked myeloid colony–forming ability (data not shown). We thus focused on Lin⁻Flt3⁺ cells with intermediate expression of c-Kit (c-Kit^{int}) that included at least two distinct cell populations, Lin⁻c-Kit^{int}Flt3⁺IL-7Rα⁻M-CSFR⁺ cells and Lin⁻c-Kit^{int}Flt3⁺IL-7Rα⁺M-CSFR⁻ cells (**Fig. 1a**), and that we could sort to purity by flow cytometry (**Fig. 1b** and data not shown). As 60–80% of previously defined common lymphoid

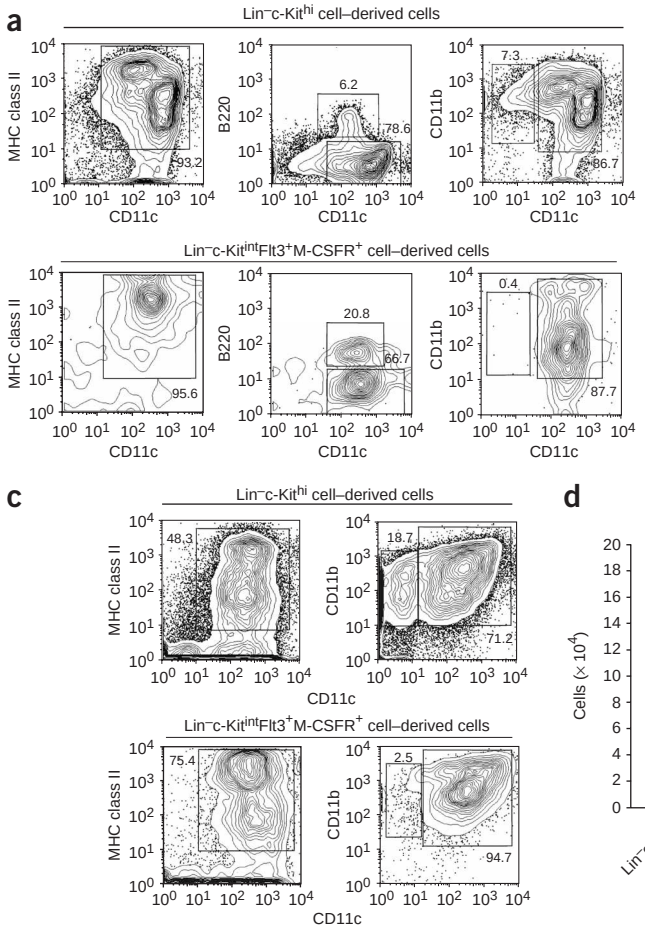

Figure 2 Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ progenitors generate pDCs and cDCs *in vitro*. Flow cytometry and quantification of differentiation potentials. (**a**) Sorted Lin⁻c-Kit^hi and Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells cultured for 8 d in media supplemented with humanFlt3L-Ig. (**b**) Total pDCs (CD11c⁺B220⁺) and cDCs (CD11c⁺B220⁻) per 2×10^4 cells plated. (**c**) Sorted Lin⁻c-Kit^hi and Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells cultured for 8 d with GM-CSF-supplemented media. (**d**) Total cDCs (CD11c⁺MHCII⁺) per 2×10^4 cells plated. Numbers adjacent to outlined areas (**a**,**c**) indicate percent cells in each. Data are representative of ten (**a**) or three (**c**) experiments or the mean + s.d. of three independent experiments (**b**,**d**).

progenitors (Lin⁻c-Kit^intSca-1^intIL-7Rα⁺) express Flt3 (refs. 16,17), the Lin⁻c-Kit^intFlt3⁺IL-7Rα⁺M-CSFR⁻ cells (**Fig. 1a**) included a major fraction of common lymphoid progenitors, and thus we used these cells in subsequent experiments as comparator lymphoid progenitors.

The Flt3⁺M-CSFR⁺ cells (**Fig. 1a**) accounted for about 0.1% of total bone marrow nucleated cells in 6- to 12-week-old mice, seemed undifferentiated, as assessed by light microscopy (**Fig. 1c**), expressed CD34, CD62L, CD24 and CD44, had low expression of AA4.1 and Ly6c, and were negative for major histocompatibility complex (MHC) class II, CD11c, CD40, CD45RA, CD69 and CD86 (**Fig. 1d** and **Supplementary Fig. 1** online). Also, a large fraction (about 40%) of these cells were in the S-G2-M phase of the cell cycle (**Fig. 1e**). Thus, Flt3⁺M-CSFR⁺ cells (**Fig. 1a**) were a relatively uniform, highly proliferating cell population.

In vitro differentiation of Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells

We evaluated the myeloerythroid and B cell developmental potential of Flt3⁺M-CSFR⁺ cells and, for comparison, multipotent and lymphoid-committed cells in permissive *in vitro* colony-forming assays. Sorted Lin⁻c-Kit^hi cells (a mixture of stem cells and progenitor cells) had myeloerythroid colony–forming potential, whereas Flt3⁺IL-7Rα⁺ cells were devoid of myeloid colony–forming potential, and less than 4% of Flt3⁺M-CSFR⁺ cells produced myeloid colonies such as macrophage colony-forming units and granulocyte-macrophage colony-forming units, and they completely lacked granulocyte colony–forming activity and megakaryocyte-erythroid colony–forming activity (**Fig. 1f**). Similarly, in the presence of M-CSF, Lin⁻c-Kit^hi cells gave rise to large percentages of macrophage colonies and less than 3% of Flt3⁺M-CSFR⁺ cells gave rise to macrophage colonies, whereas Flt3⁺IL-7Rα⁺ cells gave rise to no macrophage colonies (**Fig. 1g**). Both Lin⁻c-Kit^hi cells and Flt3⁺IL-7Rα⁺ cells formed B cell colonies, whereas Flt3⁺M-CSFR⁺ cells were devoid of B cell differentiation potential (**Fig. 1h**). On the basis of these results, we conclude that Flt3⁺M-CSFR⁺ cells have minimal, if any, myeloid differentiation potential and lack erythroid and pre–B cell differentiation potential in permissive assays *in vitro*.

To assess *in vitro* pDC and cDC development, we sorted Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells and Lin⁻c-Kit^hi cells, containing a mixture of stem and progenitor cells, and cultured the cells for 8 d in Flt3L-supplemented medium[20,26]. Lin⁻c-Kit^hi cells gave rise to CD11c⁺B220⁺ pDCs, CD11c⁺B220⁻ cDCs and some CD11c⁻CD11b⁺ myeloid cells, whereas Flt3⁺M-CSFR⁺ cells differentiated into an almost completely purely CD11c⁺MHCII⁺ cell population consisting of both CD11c⁺B220⁺ pDCs and CD11c⁺B220⁻ cDCs, at a ratio of about 1:3, reflecting the ratio in steady-state spleen (**Fig. 2a**). Lin⁻c-Kit^hi cell populations expanded about 5-fold and Flt3⁺M-CSFR⁺ cell populations expanded about 2.5-fold, and whereas both input populations produced similar pDC numbers, Lin⁻c-Kit^hi cells produced about 2.5-fold more cDCs (**Fig. 2b**). In GM-CSF-supplemented cultures, however, both populations gave rise to CD11c⁺CD11b⁺MHCII⁺ cDCs with similar efficiency (expansion of six- to sevenfold; **Fig. 2c**,**d**). Thus, these results demonstrate that Flt3⁺M-CSFR⁺ cell populations include progenitors that differentiate efficiently into pDCs and cDCs. Notably, in these culture conditions, 20% of cells derived from Lin⁻c-Kit^hi cells (a mixture of stem and progenitor cells) had a monocyte-macrophage CD11c⁻CD11b⁺ phenotype, whereas only a few cells derived from Flt3⁻M-CSFR⁺ cells had this phenotype (**Fig. 2a**,**c**).

We next tested if single cells in the Flt3⁺M-CSFR⁺ population were capable of generating both pDCs and cDCs. Although Lin⁻c-Kit^hi cells divided vigorously, the overall proliferation of Flt3⁺M-CSFR⁺ cells was limited in Flt3L-supplemented cultures (**Fig. 3a**). Thus, it was not feasible to convincingly detect progeny from single seeded cells with this assay. We therefore tested if Flt3L-supplemented stromal cell cultures might provide additional factors supporting Flt3⁺M-CSFR⁺ cell proliferation. When cultured on OP9 mouse bone marrow stromal cells, Flt3⁺M-CSFR⁺ cells divided about eight to nine times but produced only cDCs (data not shown). When cultured on mouse bone marrow stromal Ac6 cells, however, Flt3⁺M-CSFR⁺ cells divided

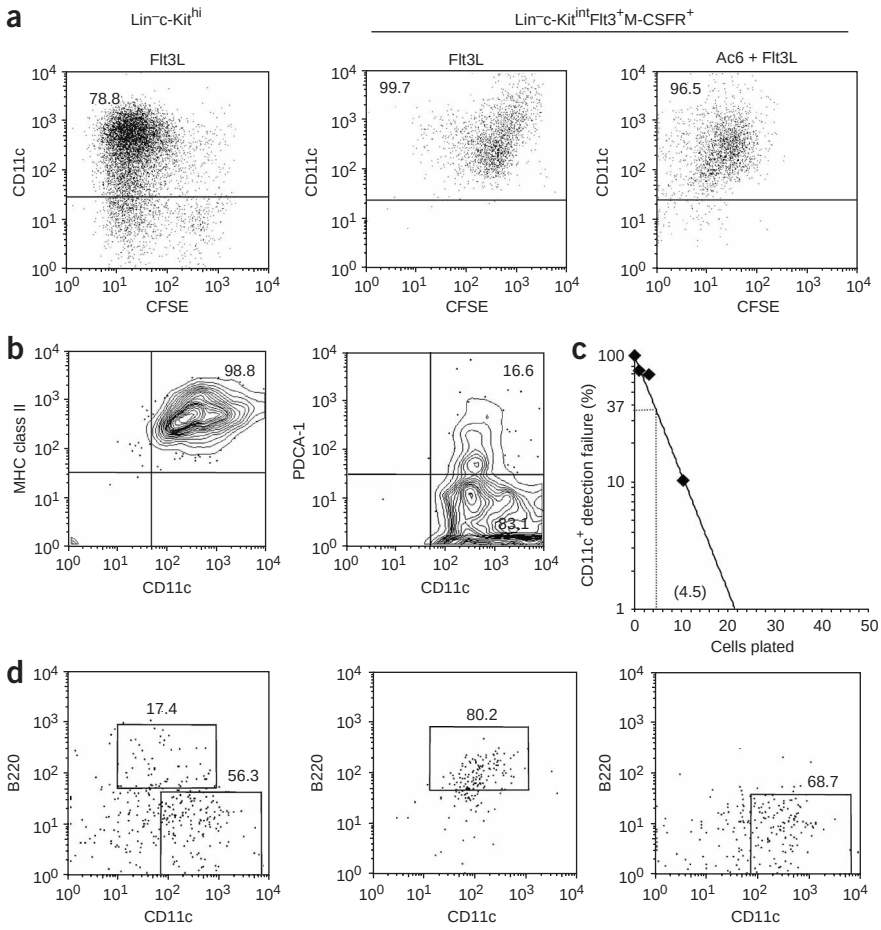

Figure 3 Single Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells generate both pDCs and cDCs. (**a**) Flow cytometry of CFSE-labeled Lin⁻c-Kit^{hi} and Lin⁻c-Kit^{int} Flt3⁺M-CSFR⁺ cells cultured for 8 d in media supplemented with human Flt3L-Ig (Flt3L), or on Ac6 stroma cells in that media (Ac6 + Flt3L), followed by analysis of CFSE dilution and CD11c expression. Numbers in plots indicate percent CD11c⁺ cells. (**b**) Flow cytometry of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells cultured for 8 d on Ac6 cells in media supplemented with human Flt3L-Ig, followed by analysis of DC cell surface marker expression. Numbers in quadrants indicate percent cells in each. (**c**) Clonal analysis of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells sorted in limiting-dilution conditions and cultured on Ac6 stromal cells in media supplemented with human Flt3L-Ig; single wells were stained and analyzed by flow cytometry on day 12. Vertical axis, detection failure for CD11c⁺ differentiation; gray lines in graph indicate the 37% negative 'readout', with the predicted CD11c⁺ progenitor cell frequency in parenthesis. Statistics were calculated on the basis of mean values of each dilution step; the correlation coefficient for curve extrapolation was $r = 0.9706$. (**d**) Clonal pDCs and cDCs (left), pDCs (middle) or cDCs (right) derived from single Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells. Data are representative of four (**a**) or three (**b**–**d**) independent experiments.

about seven times and produced both CD11c⁺PDCA-1⁺ pDCs and CD11c⁺PDCA-1⁻ cDCs (**Fig. 3a**,**b**), a finding reminiscent of the differentiation of human pDC and cDC hematopoietic progenitor cells on Ac6 cells[14].

In the culture conditions described above, 1 of 4.5 Flt3⁺M-CSFR⁺ cells gave rise to CD11c⁺ cells, as estimated by limiting-dilution analysis (**Fig. 3c**). In line with those results, 68 of 252 single sorted Flt3⁺M-CSFR⁺ cells (about 1 of 4) gave rise to CD11c⁺ cells (**Table 1**). These included 40 clones generating only CD11c⁺B220⁻ cells, 10 clones generating only CD11c⁺B220⁺ cells, and 18 clones generating both CD11c⁺B220⁺ and CD11c⁺B220⁻ cells (**Fig. 3d** and **Table 1**). These results formally demonstrate that the Flt3⁺M-CSFR⁺ population contained single cells with both pDC and cDC differentiation capacity (about 26% of all cells that generated DCs). The true DC differentiation capacity of single Flt3⁺M-CSFR⁺ cells in this assay, however, is probably underestimated. First, we used a 'cut-off' of counting only plates with 128 or more offspring cells; thus, we did not count cells with a small clone size. Second, if Flt3⁺M-CSFR⁺ cells commit to one cell type in the first cell division, it is likely that, although they are equipped with pDC and cDC potential, often they develop into only pDCs or cDCs but not into both.

As both Flt3 and M-CSFR were expressed on the population in question but M-CSF did not promote myeloid cell development *in vitro*, we tested the effects of M-CSF plus Flt3L on *in vitro* DC differentiation. Unexpectedly, the addition of M-CSF to Flt3L-containing cultures enhanced the relative and absolute differentiation of CD11c⁺B220⁺CD45RA⁺PDCA-1⁺ pDCs from Flt3⁺M-CSFR⁺ cells (**Fig. 4**) but only slightly increased cDC differentiation (**Fig. 4c**). Of note, when we cultured Lin⁻c-Kit^{hi} cells (a mixture of hematopoietic stem and progenitor cells) in these same conditions, both pDC and cDC differentiation increased (**Supplementary Fig. 2a**,**b** online). Thus, M-CSF in combination with Flt3L enhances DC differentiation, particularly the differentiation of pDCs from Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells *in vitro*.

In vivo differentiation of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells

To evaluate developmental potential *in vivo*, we injected 1×10^4 double-sorted Flt3⁺M-CSFR⁺ cells together with 2×10^5 host bone marrow cells into lethally irradiated mice and analyzed cellular offspring at various time points. At 10 d after transplantation, Flt3⁺M-CSFR⁺ cells gave rise to about 0.8% nucleated spleen cells consisting mostly of CD11c⁺MHCII⁺ DCs that included CD11c⁺CD8α⁺ and CD11c⁺CD8α⁻ (both CD4⁻ and CD4⁺) cDC subsets as well as CD11c⁺B220⁺ pDCs (**Fig. 5a** and **Supplementary Fig. 3** online). Flt3⁺M-CSFR⁺ cells also differentiated into DC subsets in bone marrow (**Fig. 5b**), whereas we rarely detected any progeny in the thymus or nonlymphoid organs such as liver (data not shown). At day 5 after injection into lethally irradiated mice, most cell progeny in spleen and bone marrow had undergone multiple divisions, as detected by dilution of CFSE (carboxyfluorescein diacetate

Table 1 Clonal pDC and cDC differentiation

Progeny phenotype	CD11c⁺	CD11c⁺B220⁺ and CD11c⁺B220⁻	CD11c⁺B220⁺ only	CD11c⁺B220⁻ only
Wells	68	18	10	40

Flow cytometry of the progeny of single Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells sorted and plated at a density of 1 cell per well in 96-well plates (for a total of 252 wells) on irradiated Ac6 stromal cells in Flt3L-supplemented media. Values represent the number of wells at day 12 with cells of each phenotype. Data are combined from three independent experiments.

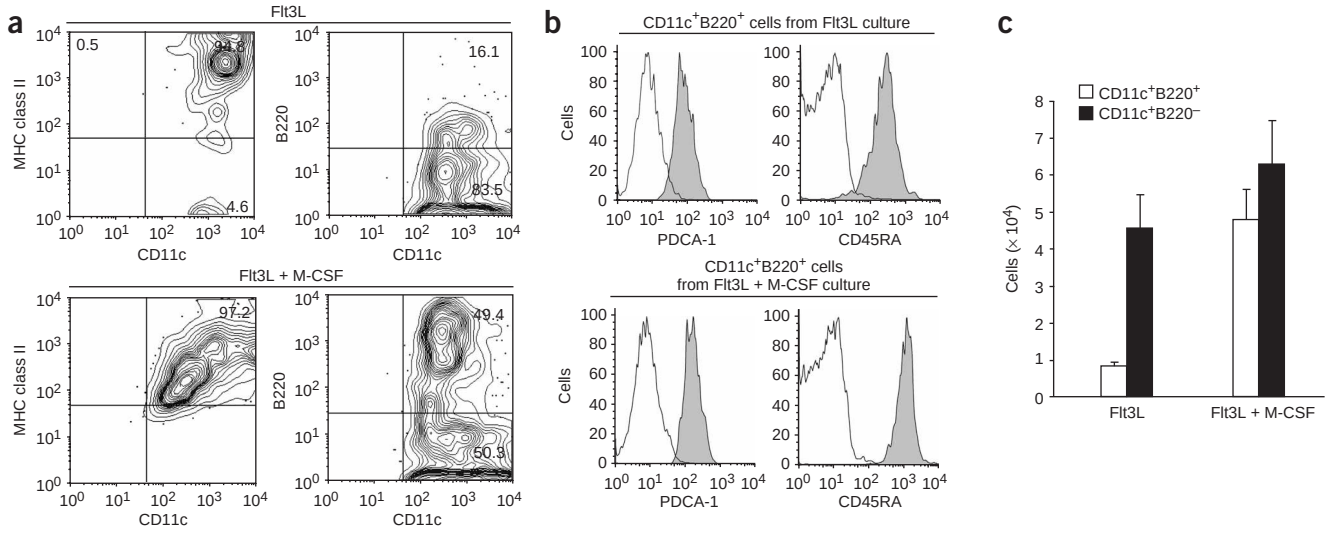

Figure 4 M-CSF acts together with Flt3L in the expansion of pDC populations from Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$ cells. (**a**) Flow cytometry of sorted Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$ cells cultured for 8 d with human Flt3L-Ig, without (top) or with (bottom) M-CSF-supplemented media. (**b**) Cell surface marker expression (shaded) and the respective isotype controls (open) on pDCs derived from Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$ cells cultured as described in **a**. (**c**) *In vitro* differentiation of Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$ cells, showing total yields of pDCs (CD11c$^+$B220$^+$) and cDCs (CD11c$^+$B220$^-$) per 2 × 10^4 precursor cells plated. Data are representative of three experiments (**a**,**b**) or the mean + s.d. of three independent experiments (**c**).

succinimidyl diester; **Fig. 5c**). Spleen progeny derived from Flt3$^+$M-CSFR$^+$ cells peaked at day 10, resulting in a population expansion of about seven- to eightfold of input cells at this time, whereas it was hardly detectable at day 21 after transplantation (**Fig. 5d**,**e** and data not shown). In line with published results[7,8,15–17], spleen DCs derived from common myeloid progenitors and Lin$^-$c-Kit^{int}Flt3$^+$IL-7Rα$^+$ lymphoid progenitors reached higher absolute numbers and were detectable as late as 21 days after transplantation, before decreasing to background numbers (**Fig. 5d** and data not shown). All progenitor fractions, however, produced spleen pDC and cDC populations at similar ratio of about 1:4 *in vivo* at all time points analyzed (**Fig. 5e** and data not shown).

Intra-animal comparison of donor- versus host-derived cells showed that all DC populations derived from Flt3$^+$M-CSFR$^+$ progenitors were very similar to host-derived DCs in phenotype and relative distribution (**Fig. 5a**,**b** and **Supplementary Fig. 4a**,**b** online). Furthermore, at day 10, spleen DCs generated from 1 × 10^4 Flt3$^+$M-CSFR$^+$ cells reached absolute numbers similar to those reached by DCs generated from 2 × 10^5 transplanted host total bone marrow cells (including about 40 HSCs, 2 × 10^4 c-Kit$^+$ multi- and oligopotent progenitors, and 2 × 10^2 Flt3$^+$M-CSFR$^+$ cells) and possibly some radiation-resistant host cells (**Supplementary Fig. 4c**). Thus, Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$ cell numbers increased rapidly to reconstitute spleen pDCs and all cDC subsets *in vivo*.

As lethal irradiation causes alterations such as upregulation of Flt3L[42] (data not shown), the conditions used above did not represent a steady-state situation and might have favored DC-biased differentiation of transferred cells. To determine if Flt3$^+$M-CSFR$^+$ cells would produce DCs in conditions mimicking steady-state development more closely, we transferred cells into sublethally irradiated or unirradiated recipient mice. Although in both settings pDCs and cDC subsets were detectable, relative and absolute progeny cell numbers decreased, being detectable only after pre-enrichment of unconditioned recipients for CD11c$^+$ or donor CD45$^+$ cells (**Supplementary Figs. 5** and **6** online and data not shown). Notably, although progeny of progenitors were not detectable in the (altered) lymph node structures of irradiated mice at time points up to 14 d after transplantation, Flt3$^+$M-CSFR$^+$ cells gave rise to both CD11c$^+$PDCA-1$^+$ pDCs and CD11c$^+$PDCA-1$^-$ cDCs in the lymph nodes of unirradiated mice (**Supplementary Fig. 5a**). Furthermore, the absolute numbers of pDCs and cDCs derived from Flt3$^+$M-CSFR$^+$ cells were enhanced in spleen and lymph nodes in unirradiated mice injected with Flt3L for 7 d after transplantation (**Supplementary Fig. 5b**). In any setting for up to 4 weeks after transplantation of Flt3$^+$M-CSFR$^+$ cells (the longest time mice were monitored), we detected no other cell lineages such as CD3$^+$, CD19$^+$ or NK1.1^{hi} lymphoid cells, CD11b$^+$CD11c$^-$ monocytes, Gr-1$^+$CD11b$^+$ granulocyte or Ter119$^+$ erythroid cells, whereas we identified all these lineages as being derived from host cells (**Fig. 5a** and **Supplementary Fig. 4a**,**b**). Thus, Flt3$^+$M-CSFR$^+$ cells had potent *in vivo* reconstitution potential for the development of pDCs and all cDC subsets, whereas other cell lineage differentiation was not detectable. Notably, competitively transplanted cells did differentiate into other lineages during this period.

Flt3$^+$M-CSFR$^+$ cell–derived spleen CD11c$^+$B220$^+$ cells had typical pDC morphology (**Fig. 6a**), expressed CD45RA and Ly6/c, had low expression of CD11b (**Fig. 6b** and data not shown) and, after *in vitro* stimulation with CpG oligodeoxynucleotide, transcribed interferon-α mRNA at amounts similar to its transcription by wild-type pDCs (**Fig. 6c**). In contrast, Flt3$^+$M-CSFR$^+$ cell–derived spleen CD11c$^+$B220$^-$ cells had typical cDC morphology after activation (**Fig. 6d**), had no or low expression of CD45RA, were partly positive for CD11b (**Fig. 6e**), and induced T cell proliferation in allogeneic mixed lymphocyte reactions (**Fig. 6f**). Furthermore, after stimulation with CpG oligodeoxynucleotide, Flt3$^+$M-CSFR$^+$ cell–derived splenic CD11c$^+$B220$^+$ pDCs and CD11c$^+$B220$^-$ cDCs upregulated the costimulatory molecules CD80 and CD86 as well as MHC class II (**Supplementary Fig. 7a**,**b** online). Thus, Flt3$^+$M-CSFR$^+$ cells gave rise to functional pDCs and cDCs *in vivo*.

Precursors and descendents of Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$ cells

To determine the potential progenitors of Flt3$^+$M-CSFR$^+$ cells, we injected HSC populations containing Lin$^-$c-Kit^{hi}Sca-1$^+$ cells, Flt3$^+$ myeloid progenitors or Flt3$^+$ lymphoid progenitors into the bone marrow cavities of unirradiated mice and analyzed their progeny at

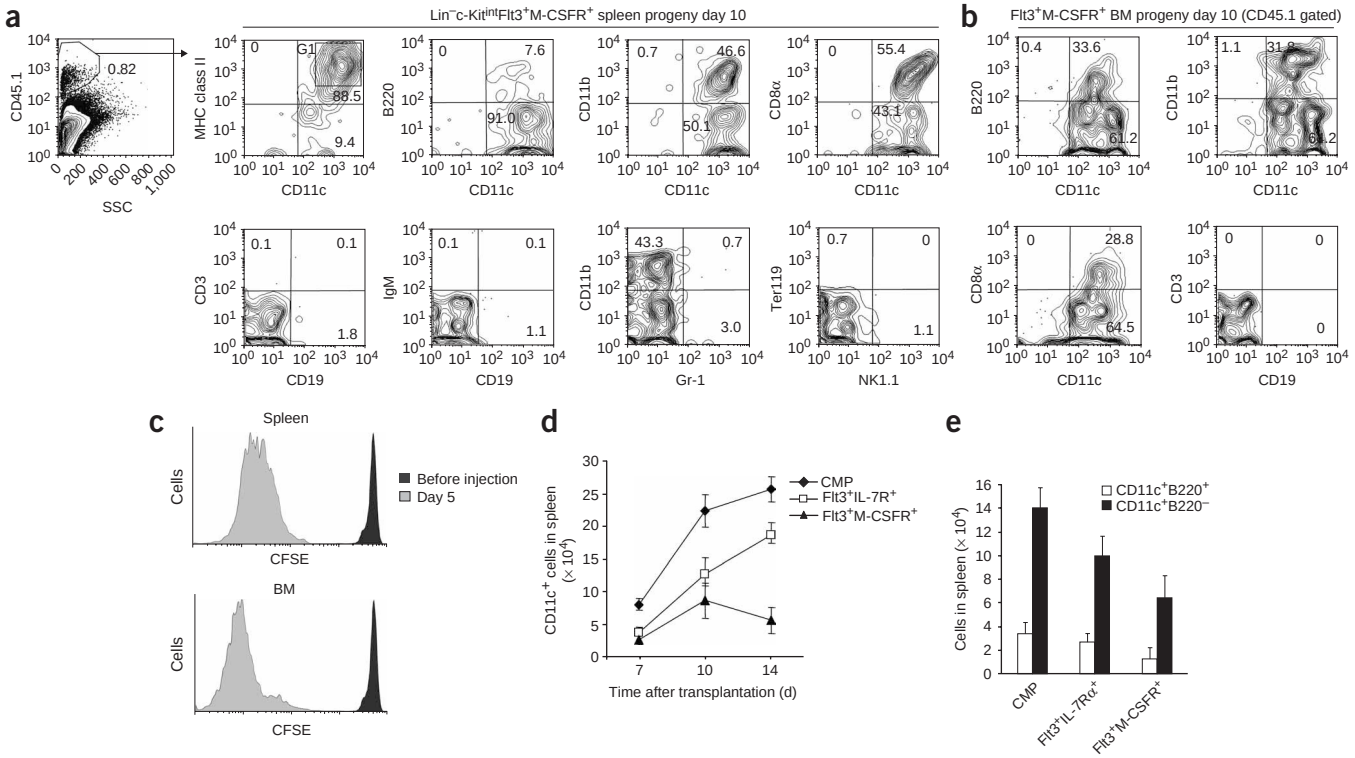

Figure 5 Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells differentiate into pDCs and cDCs in lethally irradiated mice *in vivo*. (**a**) Flow cytometry of spleen progeny at day 10 after intravenous transplantation of 1 × 10⁴ double-sorted CD45.1⁺ Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells. SSC, side scatter. (**b**) Flow cytometry of CD45.1⁺ bone marrow progeny from Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells on day 10 after transplantation. Number adjacent to outlined area (**a**, far left) indicates percent CD45.1⁺ cells; numbers in quadrants (**a**,**b**) indicate percent cells in each. (**c**) Division of 1 × 10⁴ CFSE-labeled Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells in spleen and bone marrow before and 5 d after transplantation. (**d**) Quantitative and kinetic analysis of donor-derived spleen CD11c⁺ cells after transplantation of common myeloid progenitors (CMP), Lin⁻c-Kit^intFlt3⁺IL-7Rα⁺ cells (R2 in **Fig. 1a**) and Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells. Three mice were analyzed at each time point. (**e**) Absolute numbers of progenitor-derived spleen pDCs (CD11c⁺B220⁺) and cDCs (CD11c⁺B220⁻) at day 10 after transplantation. Data are representative of five (**a**,**b**) or three (**c**) experiments or represent the mean + s.d. of three independent experiments (**d**,**e**).

day 4. Both Lin⁻c-Kit^hiSca-1⁺ cells and Flt3⁺ myeloid progenitors generated Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ bone marrow cells, whereas only few cells with this phenotype were detectable from Flt3⁺ lymphoid progenitors (**Supplementary Fig. 8a–c** online). Thus, whereas Lin⁻c-Kit^hiSca-1⁺ cells and myeloid progenitors contribute to the development of Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells in the steady state, the contribution of lymphoid progenitors remains to be evaluated.

As DCs derived from bone marrow Flt3⁺M-CSFR⁺ cells are present in spleen and lymph nodes, bone marrow progenitors themselves, their developmental intermediates or already differentiated DCs must migrate to these organs. To determine which is the case, we identified a small fraction (less than 0.1%) of cells with a Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ phenotype in spleen that did not seem to be a distinct population (**Supplementary Fig. 9a** online). In contrast to the bone marrow Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ population, the splenic Lin⁻c-Kit^intFlt3⁺M-CSFR⁺-like cells expressed CD11c; however, like the bone marrow cells, the splenic cells did not express MHC class II or CD45RA (**Supplementary Fig. 9b**). Sorted spleen Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells cultured on Ac6 stromal cells in Flt3L-supplemented media differentiated into cells with pDC and cDC phenotypes (**Supplementary Fig. 9c**). Thus, the spleen contains DC progenitors that might represent descendants of bone marrow Flt3⁺M-CSFR⁺ cells. We did not detect similar cell populations in thymus and lymph nodes (data not shown).

We also tested the effects of *in vivo* administration of Flt3L on the frequency of Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells. After daily subcutaneous injections of 10 μg Flt3L for 4 d, absolute bone marrow Flt3⁺M-CSFR⁺ cell numbers increased 2.5-fold, whereas spleen pDC and cDC numbers increased 4.0-fold and 6.7-fold, respectively (**Supplementary Fig. 10** online). Thus, similar to results reported for lymphoid and myeloid Flt3⁺ hematopoietic progenitors[17], Flt3⁺M-CSFR⁺ cell numbers increase in response to more Flt3L *in vivo*. On the basis of all the data presented here, we propose a new model for the development of pDCs and cDCs, integrating the DC progenitor populations (**Supplementary Fig. 11** online).

Gene expression profile of Lin⁻c-Kit^intFlt3⁺M-CSFR⁺ cells

To further characterize and compare the Flt3⁺M-CSFR⁺ cell population with previously defined hematopoietic stem and progenitor cells (HSCs, common myeloid progenitors, granulocyte-macrophage progenitors, megakaryocyte erythrocyte progenitors, and lymphoid Lin⁻c-Kit^intFlt3⁺IL-7Rα⁺ progenitors), we next evaluated lineage-affiliated gene-expression profiles. In line with results obtained for cell surface protein markers used for identification by flow cytometry, Flt3⁺M-CSFR⁺ cells had high expression of Flt3 mRNA similar to that of lymphoid progenitors but had low expression of interleukin 7 receptor-α (IL-7Rα) mRNA (**Fig. 7**). Consistent with their responsiveness to the respective ligand, Flt3⁺M-CSFR⁺ cells had the highest expression of *Csf2ra* mRNA (encoding GM-CSFRα) of all populations evaluated. Furthermore, and as expected, the cells had high expression of DC development–associated mRNA, such as *Stat3*, *Sfpi1*, *Spib*, *Gfi1* and *Irf8* (encoding transcription factors), whereas we

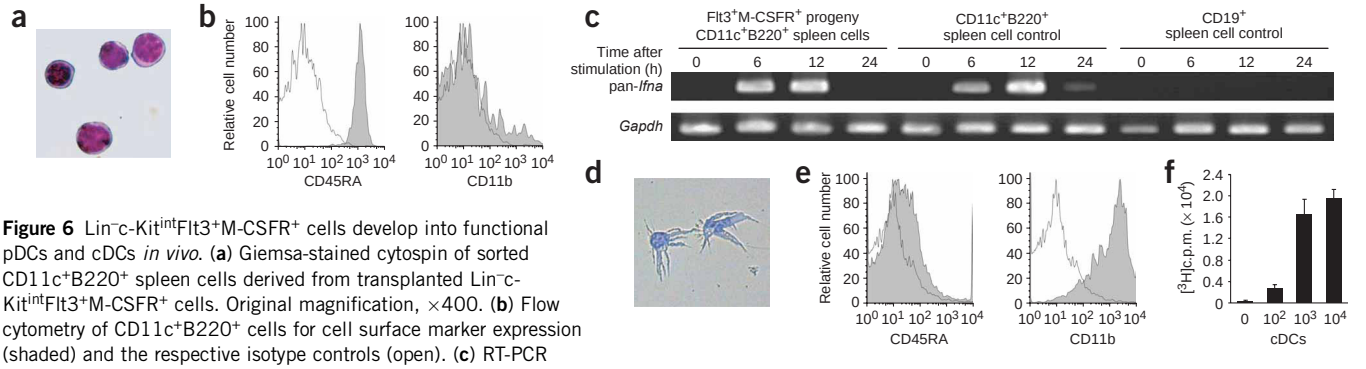

Figure 6 Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells develop into functional pDCs and cDCs *in vivo*. (**a**) Giemsa-stained cytospin of sorted CD11c⁺B220⁺ spleen cells derived from transplanted Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells. Original magnification, ×400. (**b**) Flow cytometry of CD11c⁺B220⁺ cells for cell surface marker expression (shaded) and the respective isotype controls (open). (**c**) RT-PCR analysis of the expression of *Ifna* mRNA (pan-*Ifna*) by CD11c⁺B220⁺ cells from the spleen progeny of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells, by CD11c⁺B220⁺ cells from the spleen of wild-type mice, and by CD19⁺ spleen cells, after stimulation with 2 μM CpG. *Gapdh* (encoding glyceraldehyde phosphate dehydrogenase), loading control. (**d**) Giemsa-stained cytospin of sorted CD11c⁺B220⁻ spleen cells derived from transplanted Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells cultured overnight in the presence of GM-CSF (20 ng/ml). Original magnification, ×200. (**e**) Flow cytometry of CD11c⁺B220⁻ cells for cell surface marker expression (shaded) and the respective isotype controls (open). (**f**) Thymidine-incorporation assay of proliferating allogeneic BALB/c spleen CD4⁺ T cells (2×10^5 cells) incubated with increasing numbers (horizontal axis) of sorted CD11c⁺B220⁻ spleen DCs derived from mice transplanted with Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ progenitor cells. Data are from one representative experiment of three (**a**–**e**) or represent the mean + s.d. of three independent experiments (**f**).

detected very low expression or no expression at all of erythropoiesis-associated genes such as *Gata1* and *Epor*, and the B lymphoid commitment–associated *Pax5* (**Fig. 7**). Notably, Flt3⁺M-CSFR⁺ cells had high expression of the gene encoding the chemokine-homing receptor CCR7 (**Fig. 7**). Thus, Flt3⁺M-CSFR⁺ cells had a distinct gene-expression profile fitting their developmental options described here.

DISCUSSION

Here we have investigated the longstanding issue of whether progenitors exist with both pDC and cDC differentiation capacity, but lacking alternative developmental options; that is, whether exclusive DC commitment occurs during *in vivo* hematopoiesis. The requirements for such DC-committed progenitors include the efficient generation of all DC subtypes in question, both as a population and on a single-cell level, with no production of other cell types even in the most permissive developmental assays. By testing for the expression of cytokine receptors relevant to the development of both pDCs and cDCs on Lin⁻ cells in bone marrow, the site of primary hematopoiesis in adult mice, we have identified a distinct, dividing population of Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells that account for about 0.1% of bone marrow nucleated cells and that could be isolated to purity by flow cytometry cell sorting. As a population, these cells generated pDCs as efficiently as Lin⁻c-Kit^{hi} cells did, but they generated cDCs with about 40% the efficacy of Lin⁻c-Kit^{hi} cells in Flt3L-supplemented cultures *in vitro*. In single-cell assays permissive for the development of both pDCs and cDCs, about every fourth cell generated DCs, and about one third of those generated both pDC and cDC populations, probably an underestimate of their differentiation possibilities in optimal conditions. After *in vivo* transfer, Flt3⁺M-CSFR⁺ cells efficiently generated pDCs and cDCs in bone marrow, spleen and lymph nodes even in unconditioned mice, a setting closely mimicking the steady-state situation.

Flt3⁺M-CSFR⁺ cells do not overlap by phenotype and biology with any of the progenitor cell fractions described so far[10]. Indeed, we found that they did not produce any alternative hematopoietic lineages, except that about 3% gave rise to granulocyte-macrophage colonies *in vitro*, an ability that was not detected, however, *in vivo* in conditions in which appropriate competitor cells produced these populations. Thus, the minor *in vitro* myeloid development detected might have been an ability intrinsic to some cells in the Flt3⁺M-CSFR⁺ population or might have been due to contamination of sorted cells not detectable after *in vivo* transfer.

Enforced Flt3, STAT3 or PU.1 signaling can upregulate *Flt3* expression, suggesting a self-sustaining effect of the Flt3 signaling cascade[26]. Consistent with their differentiation potential, Flt3⁺M-CSFR⁺ cells

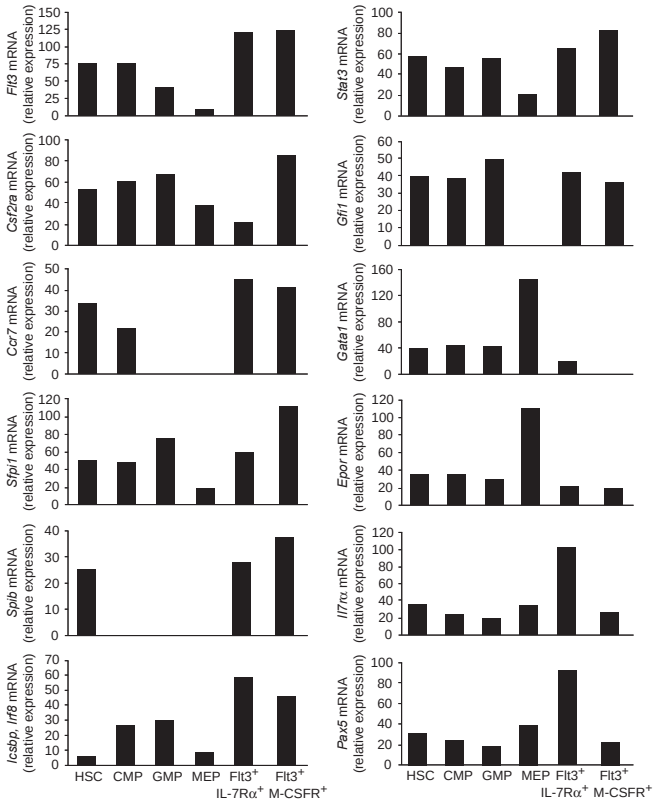

Figure 7 Expression profiles of lineage- and differentiation-affiliated genes. Real-time PCR analysis of genes encoding cytokine receptors and transcription factors in HSCs, common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), megakaryocyte erythrocyte progenitors (MEP), Lin⁻c-Kit^{int}Flt3⁺IL-7Rα⁺ cells (Flt3⁺IL-7R⁺; R2 in **Fig. 1a**) and Lin⁻c-Kit^{int}Flt3⁺M-CSFR⁺ cells (Flt3⁺M-CSFR⁺). Data are from one representative experiment of three.

had high expression of the DC development–associated genes *Stat3, Sfpi1*, *Spib*, *Gfi1* and *Irf8*, which encode transcription factors, as well as *Flt3* and *Csf2ra*, which encode cytokine receptors[26,43–45]. On the basis of these data, we conclude that Flt3$^+$M-CSFR$^+$ cells represent a phenotypically and transcriptionally distinct population of cells containing a large fraction of clonal common pDC and cDC progenitors that, by frequency and differentiation potential, are capable of maintaining steady-state DC populations in bone marrow, spleen and lymph node, whereas no other lineages are produced. We therefore call these cells 'common dendritic progenitors' (CDPs).

How can CDPs be integrated into proposed 'maps' of hematopoiesis, and how do they compare with previously described cells with DC potential in terms of frequency, phenotype, cytokine responsiveness, differentiation and proliferation capacity? CDPs might be direct descendants of Flt3-expressing multipotent myeloid and lymphoid progenitors stimulated by Flt3L[16,17,26]. Indeed, after intra–bone marrow transfer of Lin$^-$c-Kit^{hi}Sca-1$^+$ cells or Flt3$^+$ myeloid progenitors, both contributed to the differentiation of CDPs by phenotype, whereas the contribution of Flt3$^+$ lymphoid progenitors remains to be evaluated. Several other populations with DC differentiation potential have been described. These include CD11c$^+$CD31$^+$Ly6C$^+$ 'pre-immunocytes', which account for 0.5–1% of bone marrow and 1–2% of blood cells and give rise to macrophages, pDCs and cDCs without proliferation *in vitro*[36]; CD11c$^+$MHCII$^-$ 'DC precursors' (a population subsequently found to be contaminated by natural killer cells), which account for about 5% of peripheral blood nucleated cells, divide about three times after *in vivo* transfer into sublethally irradiated mice, and generate spleen pDCs and cDCs but no other cells *in vivo* with a ratio of about 1:1 for input cell to differentiated spleen cell[37]; CD11c^{int}CD45RA^{lo}CD43^{int}SIRP-α^{int}MHCII$^-$ 'pre-cDCs' in spleen, which generate all cDCs but no pDCs or other cells on a population basis *in vitro* and *in vivo*[39]; and Lin$^-$c-Kit$^+$CX3CR1$^+$ cells, which account for 0.5% of bone marrow in *Cx3cr1-gfp* 'knock in' mice, differentiate exclusively into macrophages and cDCs but not pDCs on a clonal level *in vitro* and as a population *in vivo* and have therefore been called 'macrophage and DC progenitors' (MDPs)[38].

The study of MDP cells showed that maximum MDP progeny in spleen are generated at about day 7 after sublethal irradiation and transplantation, with a ratio of about 1:10 for input cell to spleen cDC output[38]. Both MDPs and the CDPs we have described here are in the Lin$^-$ bone marrow fraction and have similar proliferation capacities, with MDPs having somewhat higher expression of c-Kit, and, at least by RT-PCR, some expression of Flt3 mRNA. However, MDPs efficiently give rise to macrophages and monocytes and lack pDC differentiation potential[38], whereas we found that CDPs exclusively generated pDCs and cDCs. Notably, and in contrast to the growth of CDPs, *in vitro* MDP growth is sustained by M-CSF as a single cytokine but not by M-CSF-deficient OP9 stromal cells supplemented with Flt3L or by Flt3L as a single cytokine[38]. Thus, CDPs by phenotype and proliferation ability are distinct from pre-immunocytes, DC precursors and pre-cDCs but might subsequently progress through cell stages in some of these probably heterogonous cell populations. In contrast to that possible sequential development, MDPs and CDPs represent two biologically different populations, one (MDPs) involved in the development of monocytes-macrophages and cDCs, and the other (CDPs) involved in the Flt3L-driven differentiation of pDCs and cDCs. It will be useful to determine the function of the receptor CX3CR1 in steady-state cDC differentiation, as mice deficient in CX3CR1 and its cognate ligand do not seem to have disturbed DC compartments[46,47]. Also, direct comparison of MDPs and CDPs in steady-state and inflammatory settings will be critical; however, the isolation of MDPs requires the use of 'reporter mice' at present.

Beyond lineal relationships, several issues regarding CDP biology remain to be clarified. CDPs expressed M-CSFR without responding efficiently to culture conditions providing only M-CSF and without producing monocyte-macrophage progeny. Both pDCs and cDCs have low expression of M-CSFR *in vivo*[34]. However, whereas deletion of the receptor or its ligand disrupts the development of monocytes-macrophages and Langerhans cells, they seem dispensable for the maintenance of steady-state lymphoid tissue DCs[32,33]. Notably, we have shown that the addition of M-CSF to Flt3L-supplemented *in vitro* cultures enhanced the development of pDCs from Lin$^-$c-Kit^{hi} cells as well as from CDPs, which suggested the involvement of M-CSF in the development of pDCs, such as in inflammatory settings, a hypothesis that remains to be tested. Also, it will be important to clarify if CDPs themselves or their respective progeny cells leave the bone marrow to home to lymphoid organs and, if they do, how this is regulated in steady-state and other conditions. The data presented here suggest that a Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$CD11c$^+$ cell population in spleen with, on a population basis, pDC and cDC differentiation ability contains direct descendents of CDPs.

Overall, on the basis of frequencies, cycling activity, cellular burst size, response to Flt3L and direct comparison to other known progenitor populations, we conclude that CDPs probably present the main intermediate DC progenitor population involved in the continuous steady-state regeneration of at least bone marrow, spleen and lymph node DCs, independently of monocyte-macrophage differentiation. CDPs will be useful in determining critical 'instructive' events necessary for DC differentiation and, ultimately, pDC and cDC commitment; the localization of CDPs in specific microenvironments might show more about how these events occur. Because at least some conserved pattern-recognition receptors are expressed by CDPs (unpublished data), it will be useful to determine how CDPs respond to inflammatory stimuli. Finally, characterization of the respective human counterparts of mouse CDPs might help to guide modulation of the DC compartment in prophylactic and therapeutic clinical settings.

METHODS

Mice. C57BL/6 (CD45.2), C57BL/Ka-Thy1.1 (CD45.1) mice were maintained at the animal facility of the Institute for Research in Biomedicine. Animals were treated in accordance with animal regulations of the Swiss Federal Veterinary Office guidelines.

Cell sorting. Bone marrow samples were immunomagnetically pre-enriched for c-Kit$^+$ cells with allophycocyanin-conjugated antibody to c-Kit (ACK2; eBioscience) and allophycocyanin microbeads (Miltenyi Biotec). Cells were then stained with phycoerythrin-indodicarbocyanine–conjugated antibodies to lineage antigens (CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), CD19 (MB19-1), CD11b (M1/70), Gr-1 (RB6-8C5), Ter119 (Ter119) and NK1.1 (PK136; Becton Dickinson)), fluorescein isothiocyanate–conjugated antibody to CD127 (A7R34), phycoerythrin-conjugated antibody to CD135 (A2F10.1) and biotin-conjugated antibody to CD115 (AFS-98; all from eBioscience except anti-NK1.1). Streptavidin-conjugated phycoerythrin-indotricarbocyanine was used for secondary labeling. Candidate DC progenitors were sorted as Lin$^-$c-Kit^{int}Flt3$^+$M-CSFR$^+$ cells, and lymphoid progenitors were sorted as Lin$^-$c-Kit^{int}Flt3$^+$IL-7Rα$^+$ cells. HSCs, common myeloid progenitors, granulocyte-macrophage progenitors, megakaryocyte erythrocyte progenitors, pDCs and cDCs were stained and sorted as described[26]. For further cell sorting and phenotypic analysis, additional monoclonal antibodies to the following were used: CD34 (RAM34), MHC class II (I-A and I-E; M-15/114.15.2), CD11c (N418), CD62L (MEL-14), AA4.1 (AA4.1), CD24 (M1/69), CD40 (1C10),

CD44 (IM7), CD45.1 (A20), CD69 (H1.2F3), CD86 (GL1), Ly6C (AL-21), immunoglobulin M (IgM; eB121-15F9; all from eBioscience); PDCA-1 (JF05-1C2.4.1; Miltenyi Biotec); and Sca-1 (E13-161.7) and CD45RA (14.8; both from Becton Dickinson). Cells were sorted analyzed with a FACSAria and a FACSCanto (Becton Dickinson Immunocytometry Systems).

***In vitro* myeloid, lymphoid, and dendritic cell differentiation assays.** For evaluation of myeloid colony formation, sorted progenitor cells were cultured in MethoCult M3134 medium (StemCell Technologies) supplemented with mouse stem cell factor, mouse IL-3, mouse IL-11, mouse GM-CSF, mouse thrombopoietin (all 10 ng/ml; all from R&D Systems), human erythropoietin (1 U/ml; Roche) and human Flt3L-Ig fusion protein (100 ng/ml)[26,48]. For analysis of macrophage colony formation, double-sorted cells were cultured in M3134 supplemented with M-CSF (10 ng/ml; R&D Systems). For B lymphoid colony formation, cells were cultured in M3134 supplemented with mouse IL-7 (10 ng/ml; R&D Systems) and/or mouse stem cell factor (10 ng/ml). Colonies were assessed and counted under an inverted microscope from day 3 to day 8. For confirmation of colony types, colonies were picked in some cases with fine-drawn Pasteur pipettes, were spun down on slides, were stained with Giemsa and were evaluated by light microscopy. For the differentiation of pDCs and cDCs, sorted cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% (vol/vol) FCS, 2-mercaptoethanol (50 μM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and human Flt3L-Ig (100 ng/ml) or mouse GM-CSF (20 ng/ml) and/or M-CSF (10 ng/ml; R&D Systems). Half of the medium was replaced every 3 d with cytokines added.

Stromal cell coculture and limiting-dilution analysis. OP9 or Ac6 cells (3×10^4) were seeded into 24-well plates 1 d before coculture. Ac6 cells were irradiated with 20 Gy immediately before plates were seeded with progenitors. $Lin^-c\text{-}Kit^{hi}$ or $Lin^-c\text{-}Kit^{int}Flt3^+M\text{-}CSFR^+$ cells were sorted, were labeled for 8 min at 37 °C with 2 μM CFSE (Molecular Probes) as described[14] and were cultured together with stromal cells in Iscove's modified Dulbecco's medium with 10% (vol/vol) FCS, supplemented with human Flt3L-Ig (100 ng/ml). For limiting-dilution assays, Ac6 stromal cells were seeded in 96-well flat-bottomed plates (3×10^3 cells per plate) at 1 d before coculture. Progenitors were double-sorted directly into the plates on irradiated Ac6 stromal cells at a density of 100, 50, 10, 2 or 1 cell per well. Cells were cultured in Iscove's modified Dulbecco's medium with 10% (vol/vol) FCS, supplemented with human Flt3L-Ig (100 ng/ml), and were analyzed on day 12. The frequency of progenitors developing as pDCs and cDCs was determined by flow cytometry and Poisson statistics. Only wells containing at least 128 cells were considered positive.

***In vivo* reconstitution assays.** Double-sorted $Lin^-c\text{-}Kit^{int}Flt3^+M\text{-}CSFR^+$ cells (1×10^4 to 2×10^4), $Lin^-c\text{-}Kit^{int}Flt3^+IL\text{-}7R\alpha^+$ cells (2×10^3) or common myeloid progenitors (1×10^4) from C57BL/Ka-Thy-1.1 (CD45.1) mice were injected intravenously into CD45.2 congenic mice that were lethally irradiated (two doses of 6 Gy with a 4-hour interval, from a Cesium 137 source; Biobeam 8000; STS), sublethally irradiated (one dose of 6 Gy) or unirradiated. For mice given lethal irradiation, 2×10^5 recipient-type total bone marrow cells were added to the injections. Mice were killed on day 7, 10, 14 or 21 or 4 weeks after transplantation. For analysis of the development of $Lin^-c\text{-}Kit^{int}Flt3^+M\text{-}CSFR^+$ cells, $Lin^-c\text{-}Kit^{hi}Sca\text{-}1^+$ cells (5×10^4 to 5.8×10^4), $Lin^-Sca\text{-}1^-c\text{-}Kit^{hi}Flt3^+$ myeloid progenitors (2.7×10^4 to 7.5×10^4) or $Lin^-c\text{-}Kit^{int}Flt3^+IL\text{-}7R\alpha^+$ lymphoid progenitors (1.6×10^4 to 5.6×10^4) from C57BL/Ka-Thy-1.1 (CD45.1) mice were transplanted into the tibia bone marrow cavities of unirradiated CD45.2 congenic mice in a volume of 10 μl of PBS, with a 30-gauge syringe. Offspring cells were assessed at day 4; they were isolated as described[26] and evaluated by flow cytometry.

Mixed lymphocyte reactions. Sorted $CD11c^+B220^-$ cells (cDCs) from the spleen progeny of mice transplanted with $Flt3^+M\text{-}CSFR^+$ cells were cultured together with 2×10^5 immunomagnetically selected (CD4 microbeads; Miltenyi Biotec) BALB/c spleen $CD4^+$ T cells in a final volume of 200 μl RPMI 1640 medium supplemented with 10% (vol/vol) FCS. Cells were cultured for 5 d and were pulsed with 1 μCi [^{3}H]thymidine per well (Amersham Biosciences) during the final 16 h of culture. Incorporation of [^{3}H]thymidine was measured on a β-plate counter (MicroBeta TriLux; EG&G Wallac).

RT-PCR analysis. For analysis of interferon-α mRNA expression, sorted $Flt3^+M\text{-}CSFR^+$ spleen progeny $CD11c^+B220^+$ pDCs, and $CD11c^+B220^+$ pDCs and $CD19^+$ cells from wild-type spleen were stimulated for 6–12 h with 2 μM CpG oligodeoxynucleotide (ggTGCATCGATGCAgggggG; lower case indicates bases with phosphorothioate-modified backbones). Total RNA was extracted with TRIzol reagent (Invitrogen) followed by treatment with DNase I (Invitrogen), then cDNA was synthesized with random hexamers and SuperScript II reverse transcriptase and was analyzed by RT-PCR with 'pan-interferon-α' primers (*Ifna2*, *Ifna4*, *Ifna5*, *Ifna6* and *Ifna8*)[49]. For quantitative mRNA expression analysis, progenitors were sorted as described[26] and cDNA was synthesized as described above. For real-time PCR, cDNA products equivalent to the RNA from 5×10^2 progenitor cells were amplified with a 7900HT Fast Real-Time PCR system (Applied Biosystems). Data were normalized to the expression of 18S rRNA by each sample. Taqman probes were from Applied Biosystems.

***In vivo* Flt3L injection.** Human Flt3L-Ig (10 μg in 100 μl PBS) or PBS alone was subcutaneously injected daily on days 1–4 and mice were analyzed at day 5.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

N.O., A.O.-O. and M.A.S. designed experiments, did experiments, collected data and contributed to the writing of the manuscript; T.O. provided advice; D.J. did cell sorting; and M.G.M. directed the study and wrote the manuscript.

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Supplementary Figure 1
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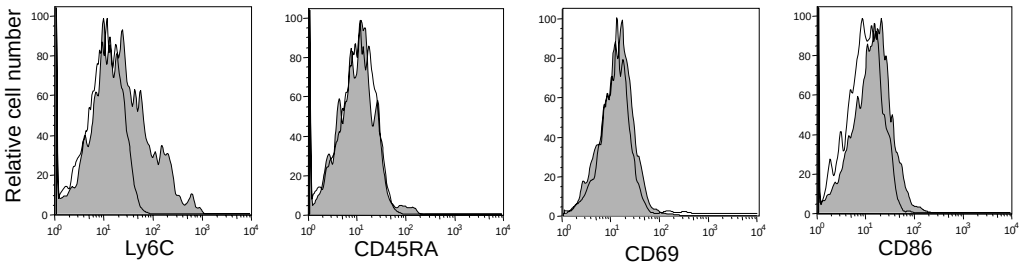

Supplementary Figure 1. Additional cell surface marker expression on lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ cells (closed histograms), and respective isotype controls (open histograms).

Supplementary Figure 2
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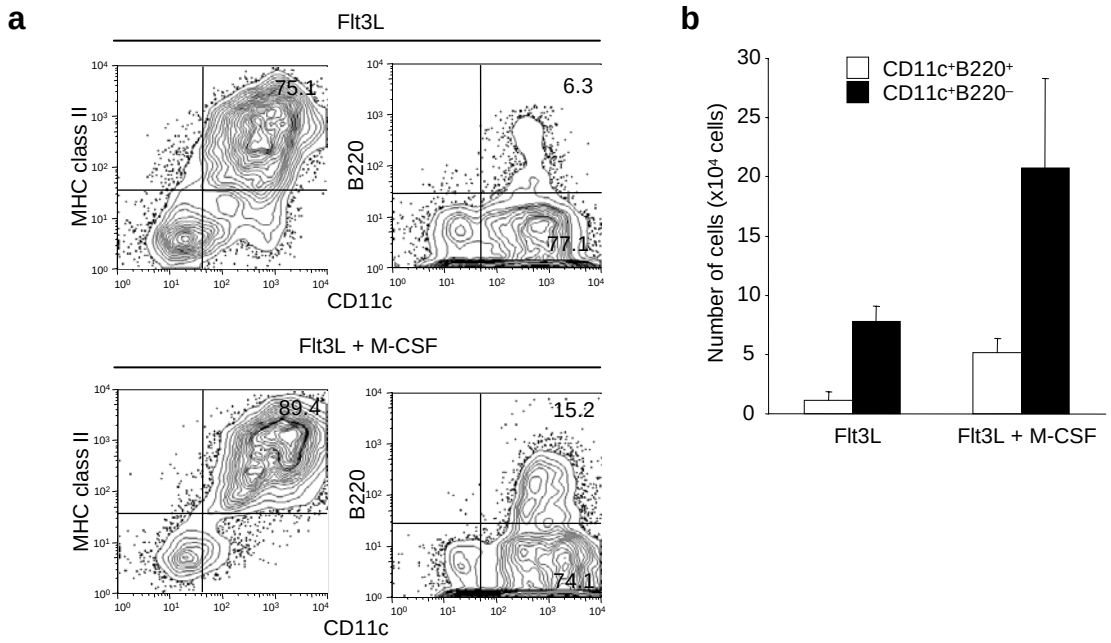

Supplementary Figure 2. M-CSF cooperates with Flt3L in the expansion of DC from lin$^-$c-kit^{high} cells. (**a**) Sorted lin$^-$c-kit^{high} cells were cultured with huFlt3L-Ig and with/without M-CSF supplemented media for 8 days. (**b**) Bars show total pDC (CD11c$^+$B220$^+$) and cDC (CD11c$^+$B220$^-$) yields per 2 x 10^4 cells plated. Data represents mean values ± s.d. from three independent experiments.

Supplementary Figure 3
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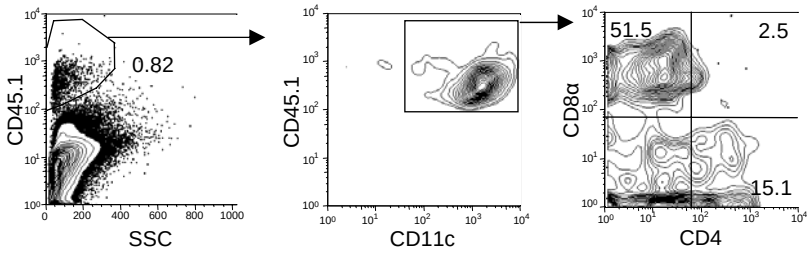
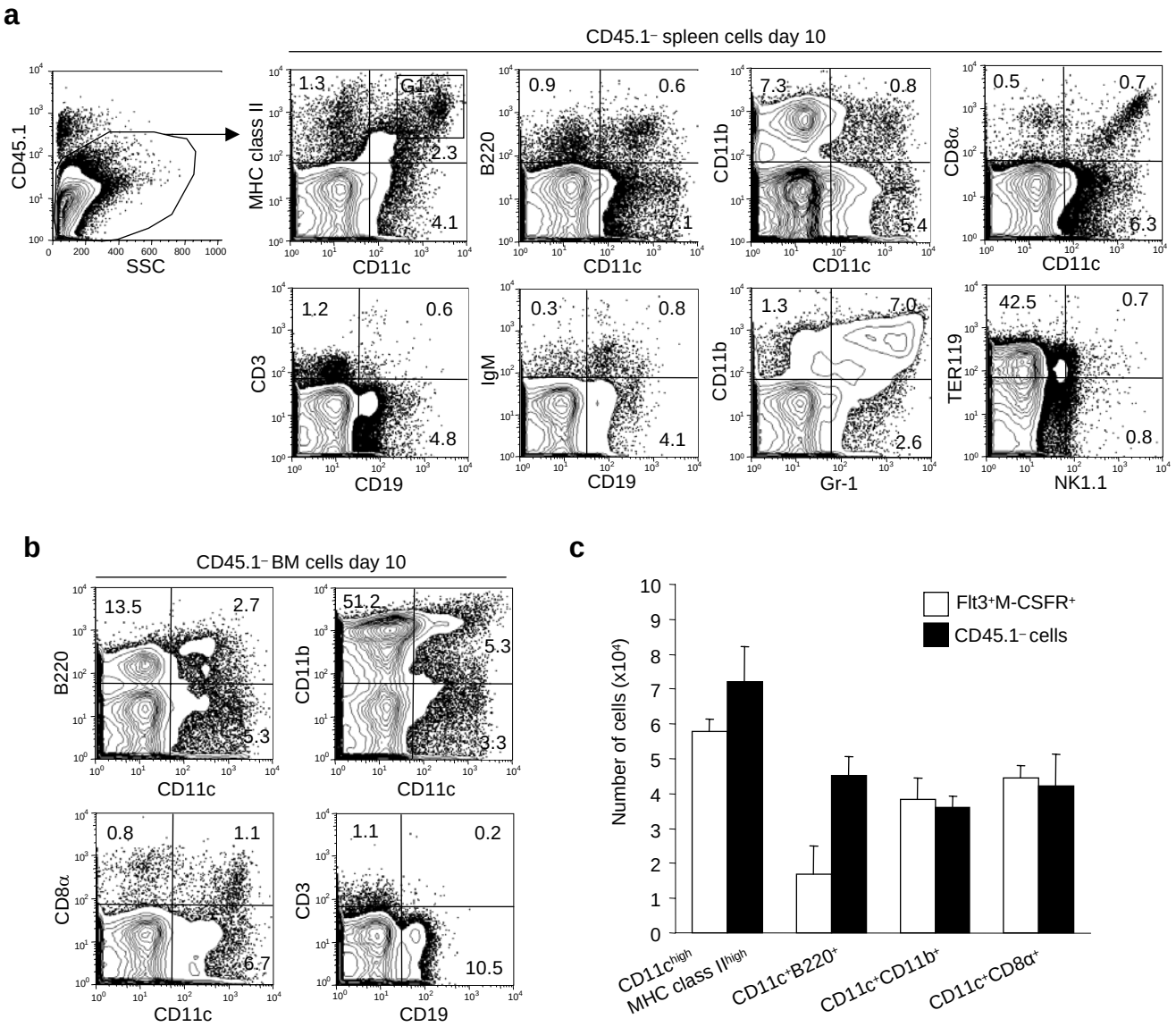


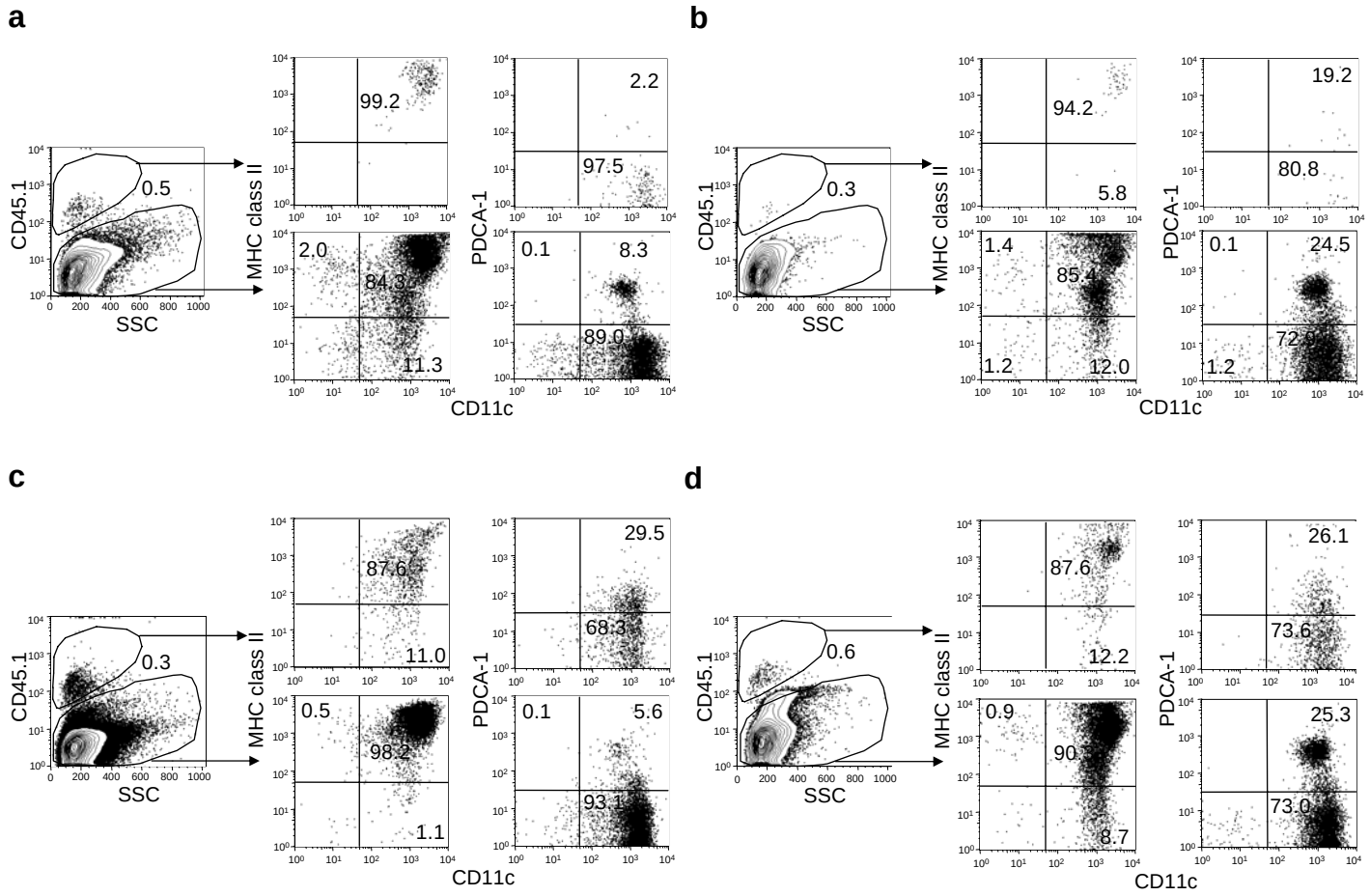
Supplementary Figure 3. Lin^{-}c-kit^{int}Flt3^{+}M-CSFR^{+} progenitor cells differentiate into resident spleen cDCs subsets *in vivo*. Representative analysis of spleen progeny at day 10 post i.v. transplantation of 1 x 10^4 double sorted CD45.1^{+} lin^{-}c-kit^{int}Flt3^{+}M-CSFR^{+} cells combined with 2 x 10^5 recipient type whole bone marrow cells into lethally irradiated mice.

Supplementary Figure 4
Onai *et al.*



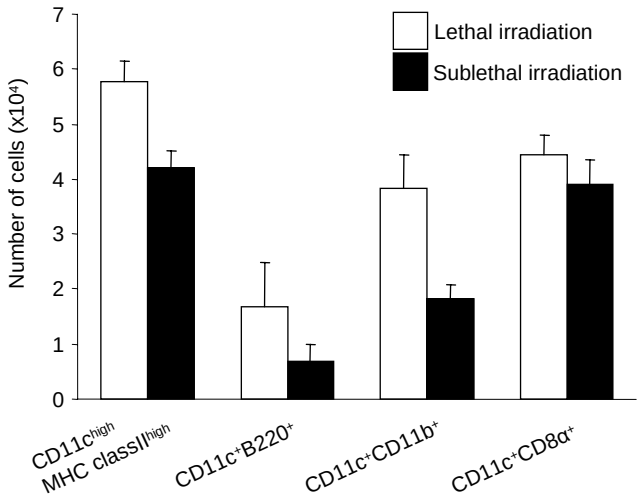
Supplementary Figure 4. (**a**) Representative analysis of CD45.1⁻ spleen cells at day 10 post i.v. transplantation of 1 x 10^4 double sorted CD45.1⁺ lin⁻c-kit^{int}Flt3⁺M-CSFR⁺ cells and 2 x 10^5 CD45.2⁺ recipient type whole bone marrow cells. (**b**) Representative analysis of CD45.1⁻ bone marrow at day 10 post i.v. transplantation of 1 x 10^4 double sorted CD45.1⁺ lin⁻c-kit^{int}Flt3⁺M-CSFR⁺ cells and 2 x 10^5 CD45.2⁺ recipient type whole bone marrow cells on day 10 post transplantation. (**c**) Bars show absolute numbers of spleen DC subsets derived from double sorted CD45.1⁺ lin⁻c-kit^{int}Flt3⁺M-CSFR⁺ cells or CD45.1⁻ cells at day 10 post transplantation. The number of CD11c^{high}MHC classII^{high} cells are calculated from gating G1 in **Fig. 5a** and respectively in **Supplementary Fig. 4a**. The data represents mean values ± s.d. from three independent experiments.

Supplementary Figure 5
Onai *et al.*

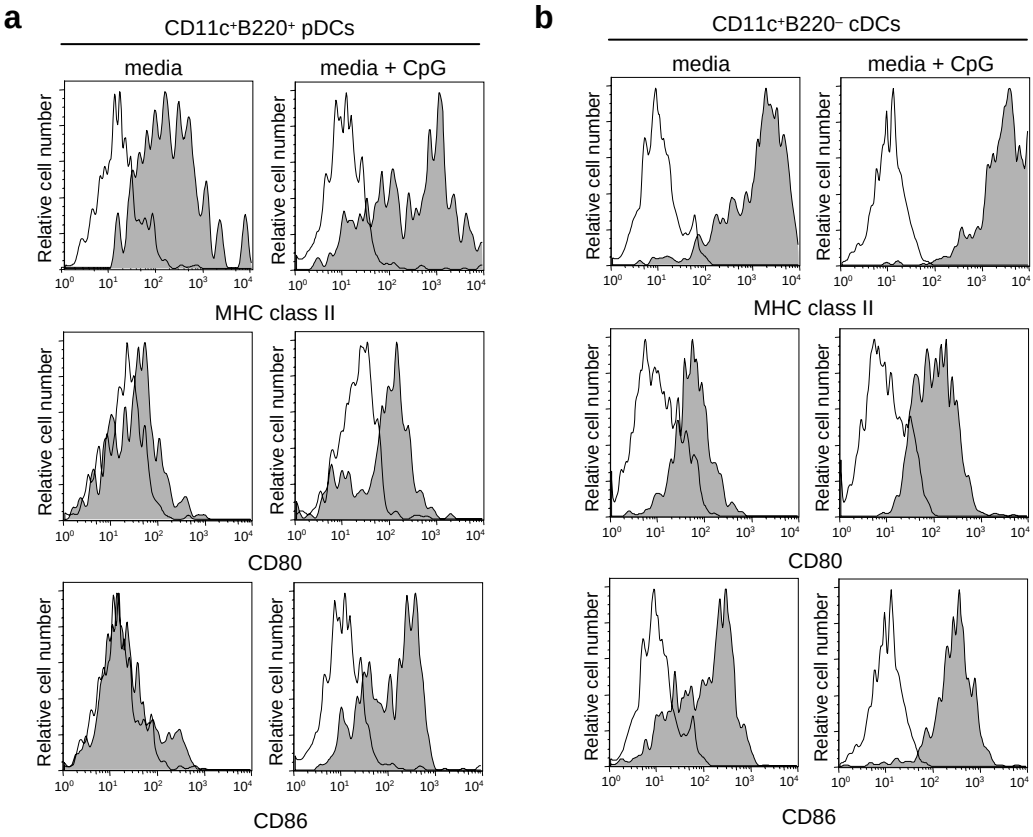


Supplementary Figure 5. Lin⁻c-kit^{int}Flt3^{+}M-CSFR^{+} cells differentiate into CD11c^{+}PDCA-1^{-} cDCs and CD11c^{+}PDCA-1^{+} pDCs in spleen and lymph nodes in non-irradiated mice and *in vivo* injection of Flt3L induces expansion of DC progeny. Double sorted lin⁻c-kit^{int}Flt3^{+}M-CSFR^{+} cells (2 x 10^4 cells) were injected intravenously into 3 week-old recipient mice. Control PBS (**a**,**b**) or hFlt3L-Ig fusion protein (100 µg in PBS) (**c,d**) was injected daily s.c. day 1-7, mice were sacrificed at day 8, and CD11c enriched cells from spleen (**a**,**c**) and lymph nodes (**b**,**d**) were subjected to FCM analysis.

Supplementary Figure 6
Onai *et al.*

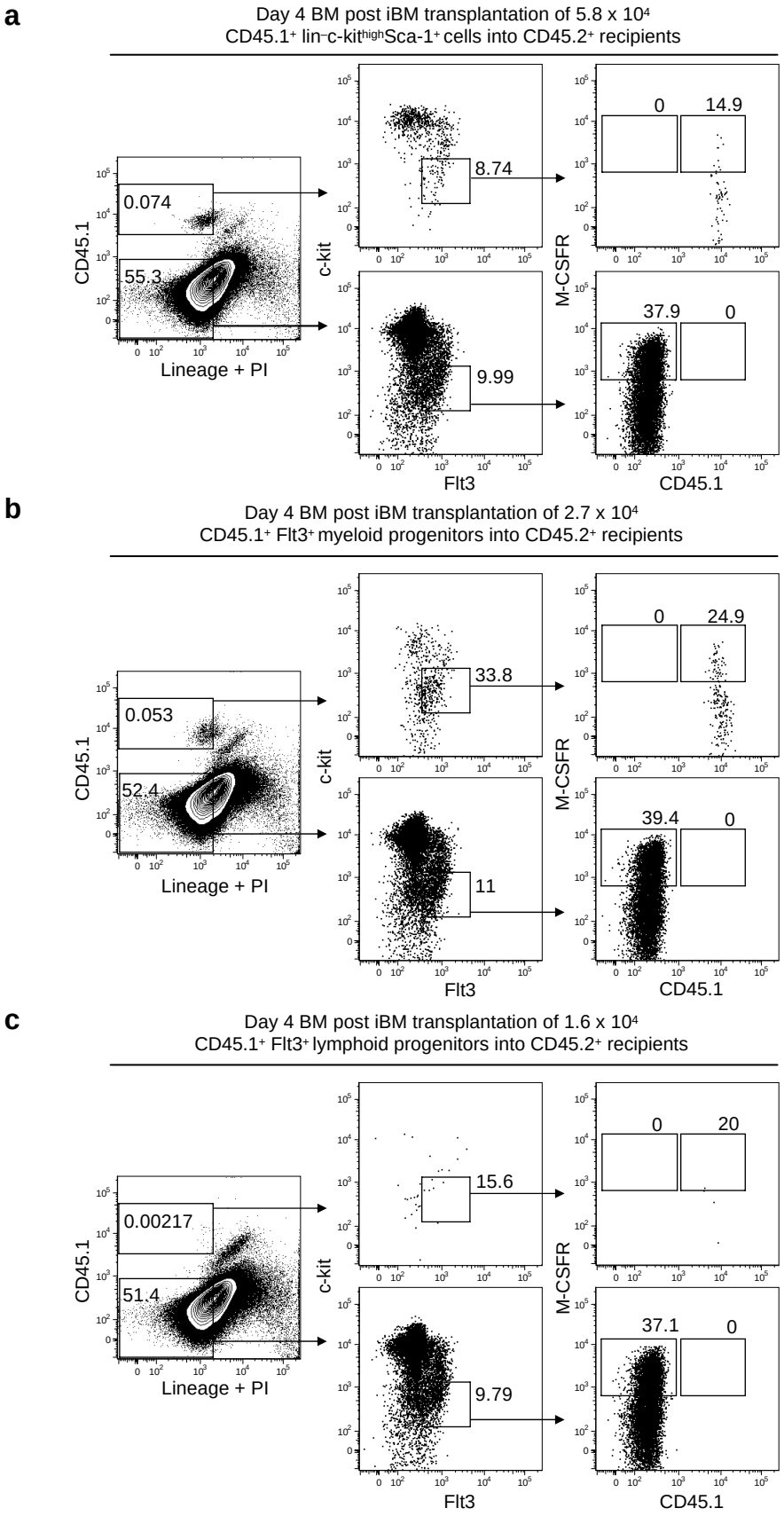


Supplementary Figure 6. Bars show absolute numbers of CD45.1$^+$ lin$^-$c-kit^{int}Flt3$^+$M-CSFR$^+$ derived spleen DC subsets in lethally and sublethally irradiated mice at day 10 post transplantation. The data represent mean values ± s.d. from three independent experiments.



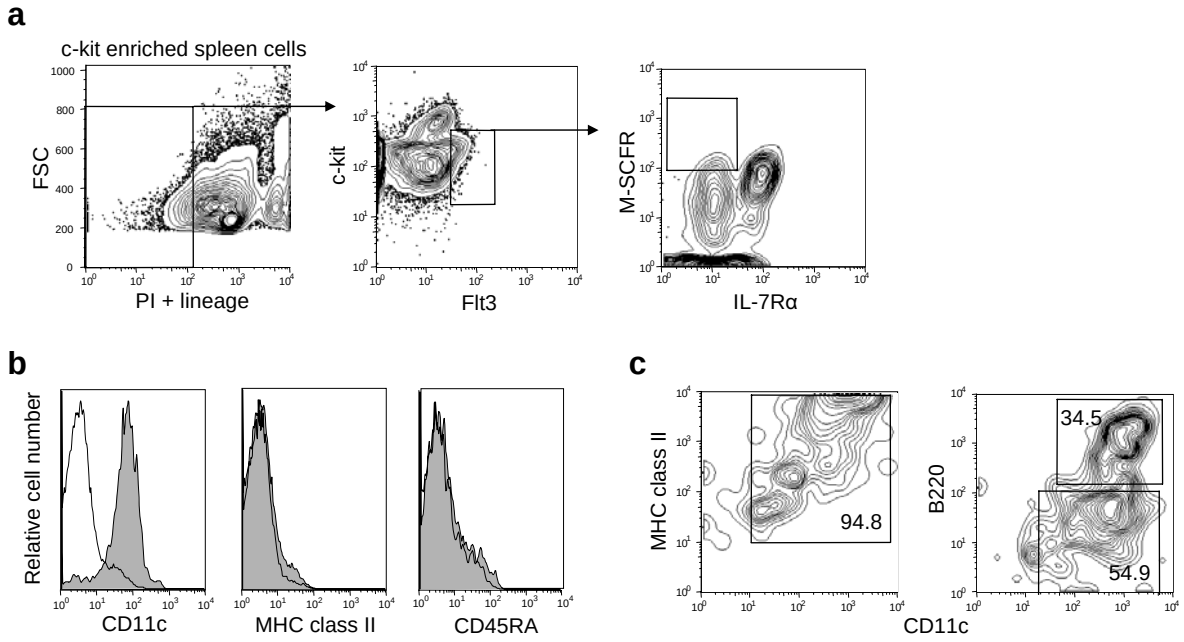
Supplementary Figure 7. Characterization of spleen CD11c^{+}B220^{+} pDCs and CD11c^{+}B220^{-} cDCs derived from lin^{-} c-kit^{int}Flt3^{+}M-CSFR^{+} cells. pDCs (**a**) and cDCs (**b**) were sorted and cultured for 24h in media +/– CpG (2μM) and subsequently analyzed. Contour plots indicate cell surface marker (MHC class II, CD80, and CD86) expression (closed histogram) or respective control antibodies (open histogram).

Supplementary Figure 8
Onai *et al.*



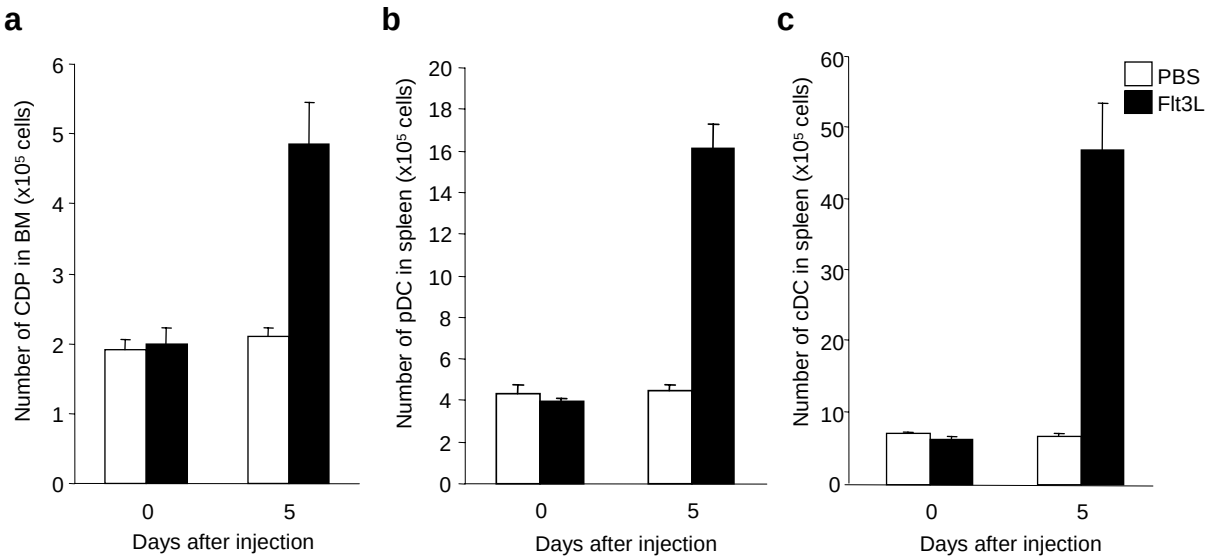
Supplementary Figure 8. Development of lin⁻c-kit^intFlt3⁺M-CSFR⁺ cells from lin⁻c-kit^highSca-1⁺ cells, Flt3⁺ myeloid progenitors, and Flt3⁺ lymphoid progenitors *in vivo*. CD45.1⁺ bone marrow progeny from lin⁻c-kit^highSca-1⁺cells (**a**), Flt3⁺ myeloid progenitors (**b**), and Flt3⁺ lymphoid progenitors (**c**) on day 4 post intra-bone marrow transplantation. Representative analysis of 2 (a), and 3 (b,c) experiments.

Supplementary Figure 9
Onai *et al.*



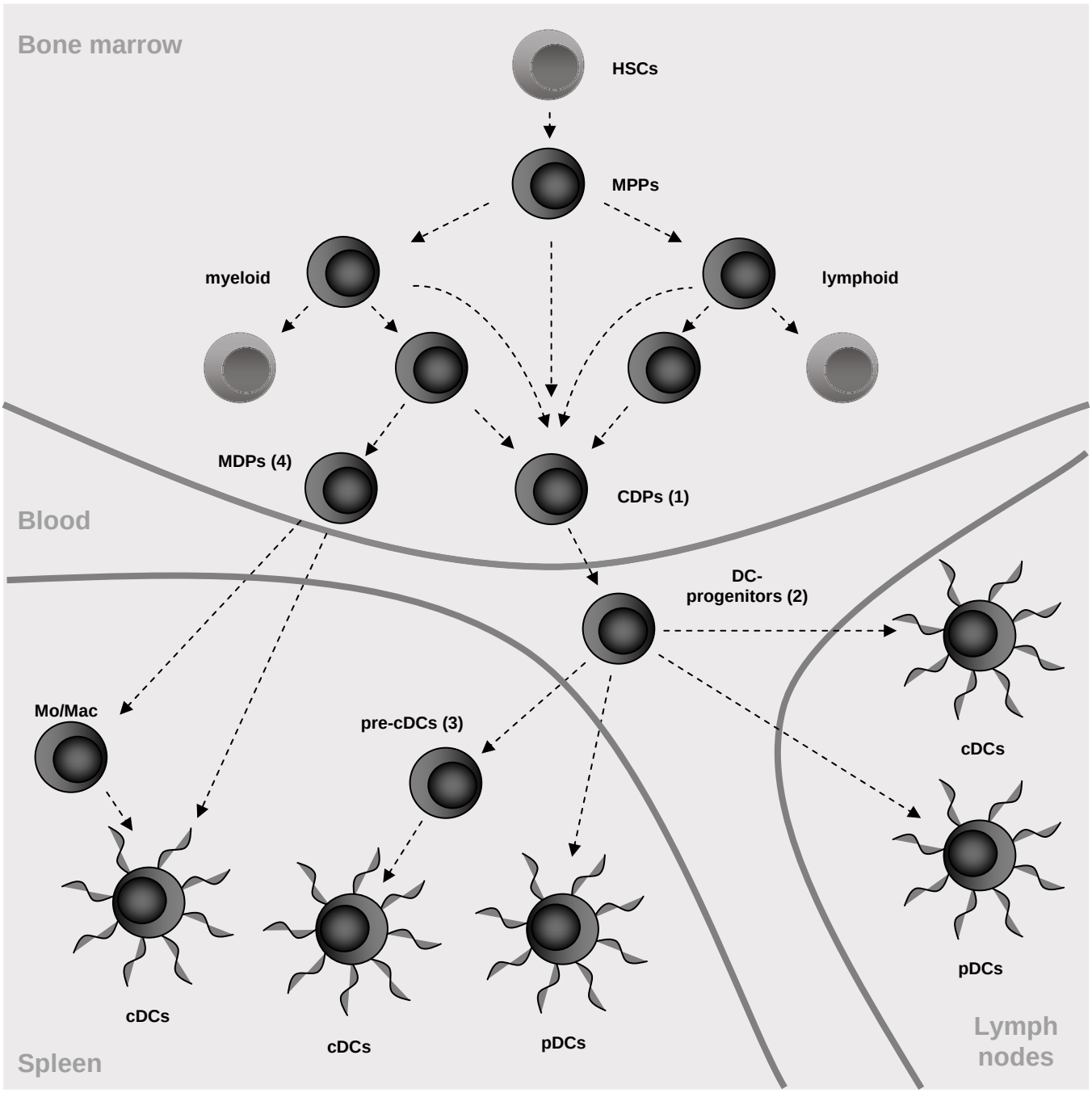
Supplementary Figure 9. Identification of lin⁻c-kit^{int}Flt3⁺M-CSFR⁺ progenitor cells in mouse spleen. **a**. The lin⁻c-kit^{int} cells in the spleen were divided into Flt3 positive and negative fractions. M-CSFR and IL-7Rα expression analysis showed the presence of lin⁻c-kit^{int}Flt3⁺M-CSFR⁺ cells. **b**. Contour plot indicate additional cell surface marker (closed histogram) and respective isotype control (open histogram) expression on lin⁻c-kit^{int}Flt3⁺M-CSFR⁺ cells. **c**. Sorted lin⁻c-kit^{int}Flt3⁺ M-CSFR⁺ cells from spleen, were cultured on Ac6 stromal cells in the presence of hFlt3L-Ig for 8 days.

Supplementary Figure 10
Onai *et al.*



Supplementary Figure 10. *In vivo* injection of Flt3L increased absolute cell number of CDPs (lin⁻c-kitintFlt3⁺M-CSFR⁺) in bone marrow (**a**), and pDC (**b**) and cDC (**c**) in spleen. hFlt3L-Ig fusion protein (100 μg in PBS) or control PBS was injected daily subcutaneously day 1-4, mice were sacrificed at day 5, and cell numbers were determined by cell counts and FCM analysis.

Supplementary Figure 11.
Onai *et al.*



Supplementary Figure 11: Model for cDC and pDC development integrating DC progenitor populations. Multipotent progenitors (MPPs), myeloid progenitors, and lymphoid progenitors might give rise to common dendritic cell progenitors (1; "CDPs"; this manuscript) in bone marrow, depending e.g. on the amount of Flt3 signal they receive (Onai *et al*., *J. Exp. Med.* 2006). CDPs could give rise to DC-progenitors (2; del Hoyo *et al*., *Nature* 2002) in blood, that then might produce pDCs and cDCs in lymphatic organs as spleen and lymph nodes. In the spleen, cDC development might proceed through pre-cDCs (3; Naik *et al*., *Nat. Immunol.* 2006). This pathway is not involved in monocyte/macrophage differentiation and might be a major Flt3-ligand regulated DC differentiation pathway in steady-state lymphoid organ DC homeostasis. Macrophage and DC progenitors (4; MDPs; Fogg *et al*., *Science* 2006) in bone marrow generate both cDCs and monocyte/macrophages, at least in spleen an possibly also in other lymphoid and non-lymphoid tissues. This pathway is not involved in pDC generation and might be a critical GM-CSF and M-CSF regulated cDC differentiation pathway tissue in cDC homeostasis and inflammatory cDC development.

Flt3 in Regulation of Type I Interferon-Producing Cell and Dendritic Cell Development

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ABSTRACT: Flt3-ligand is a nonredundant cytokine in type I interferon-producing cell (IPC) and dendritic cell (DC) development. We demonstrated that IPC and DC differentiation potential is confined to Flt3$^+$-hematopoietic progenitor cells, that Flt3-ligand drives development along both lymphoid and myeloid developmental pathways from Flt3$^+$-progenitors to Flt3$^+$-IPCs and -DCs, and that *in vivo* pharmacologic inhibition of Flt3-signaling leads to disruption of IPC and DC development in spite of consecutive Flt3-ligand upregulation in treated animals. We here summarize our recent findings that overexpression of human *Flt3* in Flt3$^-$ and Flt3$^+$ hematopoietic progenitors rescues and enhances their IPC and DC differentiation potential, respectively. Based on these data, we propose an instructive, demand-regulated, cytokine-driven IPC and DC regeneration model, where high Flt3-ligand levels initiate a self-sustaining, Flt3-STAT3 and -PU.1-mediated IPC and DC differentiation program in Flt3$^+$-hematopoietic progenitor cells.

KEYWORDS: Flt3; hematopoiesis; dendritic cells

INTRODUCTION

Hematopoiesis is regarded as a unidirectional, multilinear process where hematopoietic stem cells (HSCs) differentiate into mature hematopoietic cells by progressive loss of developmental options and restriction to one lineage, and where regeneration and expansion of specific lineages are largely regulated extrinsically by different hematopoietic cytokines.[1] However, it is unclear

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whether under physiologic conditions cytokines are capable to instruct HSCs and multipotent precursors to differentiate with lineage-restricted progenitors (extrinsic determination) or, if alternatively, HSCs and subsequent progenitors commit to lineage-restricted progenitors by intrinsic differentiation programs (intrinsic determination), and restricted progenitors are consecutively stimulated by hematopoietic cytokines, produced upon demand.[2,3] HSCs as well as multiple developmental intermediates with limited cellular expansion potential and restriction to specific mature cell types have been identified in both mice and men. These include myeloid progenitors, as clonal common myeloid progenitors (CMPs) that give rise to either granulocyte/macrophage progenitors (GMPs) or megakaryocyte/erythrocyte progenitors (MEPs),[4,5] and clonal common lymphoid progenitors (CLPs),[6,7] which produce the respective mature cell types.

Access to lineage developmental options and readiness to receive lineage-permissive and -instructive signals might be determined by relative expression levels of diverse transcription factors and cytokine receptors.[8,9] Indeed, experimental deletion or overexpression of single transcription factors is sufficient to reprogram committed progenitors or mature cells to alternative hematopoietic lineages[10] *Pax5*-deficient pre-B cells lose B cell differentiation potential and mature into T and myelomonocytic cells, however, reexpression of *Pax5* restores B cell commitment[10,11]; ectopic expression of *GATA-1* instructs HSCs and CMPs, and converts CLPs and GMPs to the megakaryocyte/erythrocyte lineage, respectively[12]; and enforced expression of *C/EBP*α and *C/EBP*β in B cells leads to macrophage differentiation.[13] Furthermore, it has been shown that *GM-CSF receptor* expression and stimulation with the cognate ligand redirect CLPs and early T cell progenitors to myeloid lineage outcomes.[14–16] The latter proves that, at least in these experimental settings, hematopoietic lineage instruction can be mediated extrinsically by cytokines.

Dendritic cells (DCs) are regulators of innate and adaptive immune responses, involved in initiation of immunity as well as in maintenance of self-tolerance.[17–19] In addition, they are cells of the hematopoietic system and are replenished from hematopoietic stem and progenitor cells.[1] In mice, multiple DC subsets that differ in maturation state, phenotype, location, and in some functions were identified.[20] For simplicity, here we will grossly divide these into $CD11c^+B220^+$ natural type I interferon-producing cells (IPCs, also called plasmacytoid cells or plasmacytoid pre-DCs) and $CD11c^+B220^-$ "conventional" DCs, consisting of $CD11c^+CD8\alpha^-CD4^-CD11b^+$, $CD11c^+CD8\alpha^-CD4^+CD11b^+$, and $CD11c^+CD8\alpha^+CD4^-CD11b^-$ subpopulations.[20] While initially it was suggested that IPCs as well as conventional $CD11c^+CD8\alpha^+$ DCs are derived from lymphoid committed progenitors,[20] it was demonstrated later that any of the IPCs and conventional DCs can be generated via lymphoid and myeloid progenitors.[21–26] Specifically, all IPCs and conventional DCs are generated by mouse CMPs, GMPs, CLPs, and pro-T1 cells, while IPC and DC differentiation potential is lost once

definitive MEPs, or B cell commitment occurs.[21–26] Thus, in contrast to other hematopoietic lineages, IPC and DC potentials are conserved along lymphoid and myeloid developmental pathways.

Flt3, a receptor tyrosine kinase with homology to c-Kit (the receptor for stem cell factor) and c-fms (the receptor for M-CSF),[27] has a nonredundant role in steady-state differentiation of IPCs and DCs *in vivo*: *Flt3-ligand* (*Flt3L*)-deficient mice and mice with hematopoietic system restricted deletion of *STAT3*, a transcription factor activated in the Flt3-signaling cascade, as well as mice that are treated with Flt3 tyrosine kinase inhibitors, show massively reduced IPCs and DCs.[28–30] On the other hand, injection or conditional expression of Flt3L in mice increases IPCs and DCs.[31–33] Furthermore, Flt3L as a single cytokine is capable to induce differentiation of IPCs and DCs in mouse bone marrow cell cultures.[34]

Flt3 is expressed in mouse short-term HSCs and multipotent progenitors,[35,36] in most CLPs and CMPs, and at lower levels on fractions of GMPs and pro-T1 cells, as well as on mature steady-state IPCs and DCs, while it is downregulated on pro-B cells, further downstream T cell progenitors, and absent on MEPs.[32,37] To determine what might define IPC and DC developmental potential in lymphoid and myeloid committed cells, we and others showed that *in vitro* and *in vivo* IPC, DC, and Langerhans cell (LC) differentiation potential is confined to Flt3-expressing hematopoietic progenitors.[32,37,38] Furthermore, we demonstrated that injection of Flt3L expands Flt3-positive, but not downstream Flt3-negative progenitors, and drives IPC and DC development along both lymphoid and myeloid differentiation pathways.[32] Based on these data, we postulated that high environmental Flt3-ligand levels and consecutive Flt3-signaling might be both, the earliest event and a continuous regulator, which determine IPC and DC developmental outcomes in bone marrow hematopoietic progenitor cells.

To test this hypothesis, we artificially expressed either *GFP* or human *Flt3-GFP* in progenitor cells using a bicistronic retroviral transduction system.[39] We showed that enforced expression of hu*Flt3* in $Flt3^-$-progenitors rescued their potential to differentiate into functional IPCs and DCs with comparable *in vitro* differentiation efficiency as $Flt3^+$-progenitors.[39] Furthermore, enforced expression of hu*Flt3* in MEPs, which under normal conditions cannot give rise to IPCs and DCs[22–26] and are contained in $Flt3^-$-progenitor cells, induced *in vitro* and *in vivo* IPC and DC differentiation, comparable to that observed from GFP^+-GMPs. Thus this data demonstrates that enforced expression and signaling of hu*Flt3* in $Flt3^-$-progenitors delivers an instructive signal to activate latent IPC and DC differentiation programs.

Enforced expression of hu*Flt3* in MEPs not only led to gain of IPC and DC developmental capacity, but, with the exception of mixed colony formation, also to gain of CFU activity of upstream myeloid progenitors, and to differentiation of erythroid and myelomonocytic cells *in vivo*. In contrast, huFlt3-signaling in GMPs did not activate megakaryocyte/erythrocyte potential. This

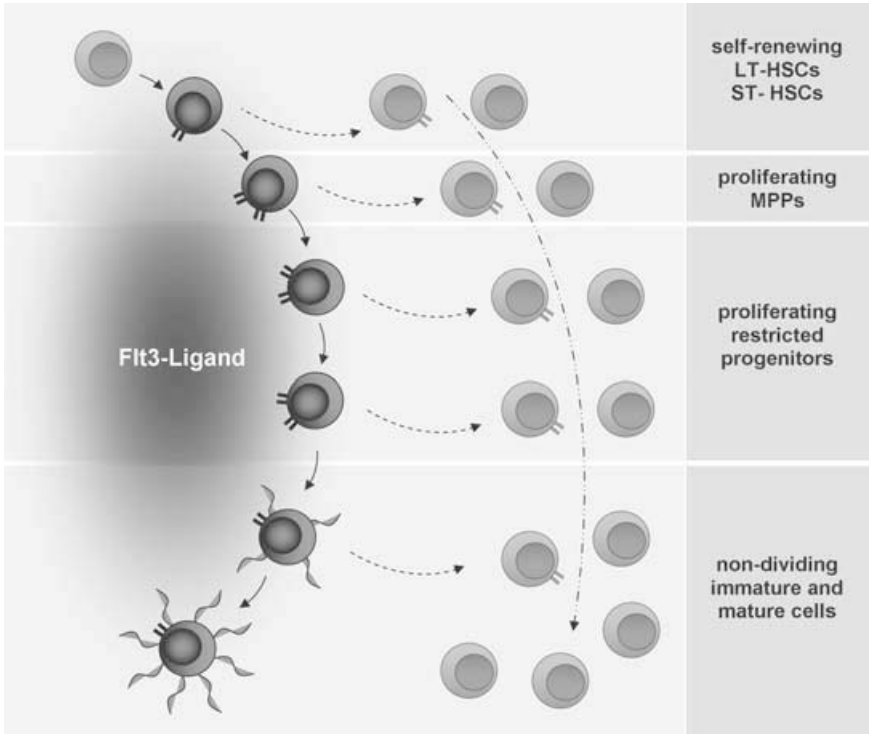

FIGURE 1. Proposed "Flt3-Licence" working model for steady-state natural IPC and DC development from early hematopoietic progenitor cells. *Bold arrows* represent continuous strong Flt3-ligand stimulation leading to IPC and DC development, *dashed arrows* represent more frequent competing signals, leading to alternative lineage outcomes.

implies that beyond activation and enhancement of IPC and DC development, Flt3-signaling is not immediate deterministic but primarily opens access to an IPC, DC, and myelomonocytic differentiation program. Thus, we propose that IPC and DC lineage outcome might be a gradual process, depending on continuous strong Flt3-signaling (FIG. 1).

What are the downstream molecular events initiated in this process by Flt3-signaling? It was shown that hematopoietic system confined deletion of *STAT3* transcription factor leads to inhibition of Flt3-driven IPC and DC development.[29] Furthermore, human *Flt3* transfection and stimulation with Flt3L in mouse myeloid 32Dcl3 cells lead to the induction of PU.1 and C/EBPα expression.[40] PU.1 cooperatively with C/EBPα activates myeloid development-associated cytokine receptor genes including *G-CSFR, M-CSFR,* and *GM-CSFR*, and these transcription factors are indispensable for granulocyte and monocyte development.[41] *PU.1*-deficient mice, in addition to other hematopoietic defects, lack either $CD8\alpha^-$ or both $CD8\alpha^-$ and $CD8\alpha^+$ DCs,

depending on the type of PU.1 deletion.[42,43] We showed that enforced huFlt3-signaling in MEPs results in enhanced expression of IPC, DC, and GM-lineage development-related transcription factors *STAT3*, *PU.1*, and *C/EBP*α, as well as expression of *G-*, *M-*, and *GM-CSF*. Thus, at least in terms of these RNA transcripts, hu*Flt3*$^+$-MEPs, but not *GFP*$^+$-MEPs, resembled the gene expression profiles of CMPs.[4,44]

Enforced expression of *STAT3* or *PU.1* in Flt3-negative MEPs was again sufficient to permit the development of both IPCs and DCs, and, most interestingly, led to the up-regulation of mouse *Flt3* mRNA levels in MEPs. This likely allowed culture supplemented, human Flt3L to cross-reactively stimulate *STAT3*- or *PU.1*-transduced cells via mouse Flt3, suggesting a self-sustaining effect of Flt3-signaling-induced *Flt3* transcription via downstream STAT3 and PU.1.

As enforced expression of hu*Flt3* in MEPs did not terminate megakaryocyte/erythrocyte differentiation potential, while hu*Flt3* expression in GMPs did not lead to gain of these differentiation potentials, how can Flt3-signaling be integrated in megakaryocyte/erythrocyte versus IPC, DC, and GM-lineage commitment? By using *PU.1*^{gfp} reporter mice, PU.1 expression was recently mapped in early hematopoietic progenitor cells. It was shown that PU.1$^+$Flt3$^+$ CMPs contain high myelomonocytic developmental potential, whereas PU.1$^-$Flt3$^-$ CMPs and PU.1$^-$MEPs have high megakaryocyte/erythrocyte potential.[3] Our data suggest that Flt3 might be critical in PU.1 regulation. GATA-1 is a nonredundant transcription factor for megakaryocyte and erythrocyte development,[3] and DNA-binding activity of GATA-1 can be suppressed by enforced *PU.1* expression, resulting in a differentiation block and apoptotic cell death of an erythroid cell line.[45] Conversely, GATA-1 inhibits binding of PU.1 to c-Jun, a co-activator of myeloid gene transactivation by PU.1.[46] Furthermore, GATA-1 interferes with DNA-binding activity of STAT3, and inhibits TPO-dependent growth of the Ba/F3 cell line.[47] Thus, as suggested previously for PU.1 and GATA-1,[48,49] relative dosage of gene transcription and protein levels will likely determine lineage outcomes. Indeed, *STAT3* and *PU.1* expression levels in hu*Flt3*$^+$-MEPs were increased to levels of normal CMPs and were somewhat lower than observed in *GFP*$^+$-, or hu*Flt3*$^+$-GMPs. Thus, MEPs with relatively lower hu*Flt3* and consecutive STAT3 and PU.1 expression do not fully inhibit GATA-1, while high Flt3-expressing and -signaling cells develop to IPC, DC, or GM lineages. In contrast, enforced expression of *STAT3* and *PU.1* in MEPs suppressed *GATA-1* and inhibited megakaryocyte/erythrocyte development. In GMPs hu*Flt3* overexpression in turn induced some *EpoR*, β-*globin*, and *GATA-1* mRNA expression; however, this was not sufficient to reactivate megakaryocyte/erythrocyte development as shown for high-level *GATA-1* expression in GMPs.[12]

Are there implications of these findings for normal hematopoiesis? Flt3 is expressed on mouse short-term (ST-) HSCs, multipotent progenitors, CLPs, CMPs, and GMPs, and *in vivo* injection of Flt3L resulted in expansion of

these cells as well as IPCs and DCs, while MEPs and their progeny remained unchanged.[31,32] Our data demonstrate that enforced Flt3 cytokine receptor signaling is sufficient to activate and also enhance IPC and DC differentiation programs, suggesting that instructive cytokine signaling might indeed occur in hematopoiesis. Thus, once Flt3-positive ST-HSCs and their offspring Flt3-positive cells are located in Flt3L-rich environments, they will likely be instructed to differentiate into IPCs and DCs (FIG. 1), a process that might be enhanced by a self-sustaining regulatory loop where Flt3 downstream transcription factors STAT3 and PU.1 in turn maintain *Flt3* receptor expression. As Flt3-signaling does not immediately silence other developmental options, and IPCs and DCs in fact only account for a minor fraction of hematopoietic cells, most Flt3-expressing progenitors will not continuously be stimulated via Flt3L but will receive and activate alternative signals, and thus consecutively acquire different myeloid or lymphoid cell fates. Our data thus support a "Flt3-permissive" developmental model, where Flt3-expressing progenitors maintain IPC and DC differentiation options in response to Flt3L as long as no competing signal shuts these down (FIG. 1).

Building on these findings, it will be important to test whether downstream dividing Flt3-positive common IPC and DC progenitors with silenced alternative developmental programs exist (common dendritic cell progenitors), and if so, which critical factors are involved in final IPC or DC lineage termination. Furthermore, it will be interesting to evaluate how $Flt3^+$ cells and Flt3-ligand-expressing cells localize in bone marrow and secondary hematolymphoid tissues, how Flt3-ligand production is regulated in steady-state and inflammatory or other hematopoietic challenge conditions, and finally, if observations made in experimental mice reflect conditions of IPC and DC development in humans.

ACKNOWLEDGMENT

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30. TUSSIWAND, R. *et al.* 2005. Inhibition of natural type I IFN-producing and dendritic cell development by a small molecule receptor tyrosine kinase inhibitor with Flt3 affinity. J. Immunol. **175:** 3674–3680.
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35. ADOLFSSON, J. *et al.* 2001. Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity **15:** 659–669.
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39. ONAI, N. *et al.* 2006. Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development. J. Exp. Med. **203:** 227–238.
40. MIZUKI, M. *et al.* 2003. Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations. Blood **101:** 3164–3173.
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43. GUERRIERO, A. *et al.* 2000. PU.1 is required for myeloid-derived but not lymphoid-derived dendritic cells. Blood **95:** 879–885.
44. MIYAMOTO, T. *et al.* 2002. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. Dev. Cell **3:** 137–147.

45. ZHANG, P. *et al*. 2000. PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. Blood **96:** 2641–2648.
46. NERLOV, C. *et al.* 2000. GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. Blood **95:** 2543–2551.
47. EZOE, S. *et al*. 2005. GATA transcription factors inhibit cytokine-dependent growth and survival of a hematopoietic cell line through the inhibition of STAT3 activity. J. Biol. Chem. **280:** 13163–13170.
48. KULESSA, H., J. FRAMPTON & T. GRAF. 1995. GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. Genes Dev. **9:** 1250–1262.
49. DEKOTER, R. P. & H. SINGH. 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. Science **288:** 1439–1441.

Curriculum vitae

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Born	8th of May 1979 in Füssen, Germany
Nationality	German

Education

PhD Graduate Studies

Institute for Research in Biomedicine
Bellinzona, Switzerland

Faculty for Biology
Eberhard Karls University Tübingen, Germany

February 2006 – present

"Dendritic cell development in steady state and inflammation"

Advisors: Prof. Dr. Markus G. Manz,
Prof. Dr. Hans-Georg Rammensee

International PhD Program in Immunology, Cell Biology and Biochemistry, Institute for Research in Biomedicine

Undergraduate Studies

University of Stuttgart
Germany

Oct 1999 – Dec 2005

Technical Biology – Diplom (Masters equivalent)
Final grade: With honour
Grades: With honour, 1-very good, 2-good, 3-satisfying, 4-adequate, 5-failed

Main subject	Technical Biochemistry & Virology	1.0
Minor subjects	Immunology	1.0
	Industrial Genetics	1.0

University of New South Wales
Sydney, Australia

July 2002 – Jan 2003

Study Abroad Program, Australia

Subjects
Immunology II, Viruses & Diseases, Biopharmaceuticals

High school
Füssen, Germany

1989 – 1998

High school degree, "Abitur"

Final grade: 1.3
Grades: 1-very good, 2-good, 3-satisfying, 4-enough, 5-insufficient, 6-failed

Main subjects Mathematics & Biology

Research experience & Publications

Division of Hematology
Eberhard-Karls-University Tübingen, Medical School

Tübingen, Germany

February 2006 - July 2006

Institute for Research in Biomedicine

Bellinzona, Switzerland

August 2006 - August 2009

Division of Hematology,
University Hospital Zürich
University of Zürich

Zürich, Switzerland

September 2009 - present

PhD Thesis

"Dendritic cell development in steady state and inflammation"
Advisors: Prof. Dr. Markus G. Manz,
Prof. Dr. Hans-Georg Rammensee

Publications

Schmid MA, Kingston D, Manz MG. Direct sensing of Toll-like receptor agonists by common dendritic progenitors leads to CXCR4 down-regulation, CCR7 upregulation and increased numbers of dendritic cells in inflamed lymph nodes.
Manuscript in preparation.

Ziegler P, Boettcher S, Schmid MA, Garavaglia G, Takizawa H, and Manz MG. Bone marrow stromal cells sense TLR4 agonists and subsequently enhance myelopoiesis.
Manuscript in preparation.

Schmid MA, Kingston D, Boddupalli S, Manz MG.
Instructive cytokine signals in dendritic cell lineage commitment.
2010 Immunol Rev 234(1):32-44.

Onai N, Manz MG, Schmid MA. Isolation of common dendritic cell progenitors (CDP) from mouse bone marrow.
2010 Methods Mol Biol 595:195-203.

Kingston D, Schmid MA, Onai N, Obata-Onai A, Baumjohann D, Manz MG. The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis.
2009 Blood 114(4):835-43.

Onai N, Obata-Onai A, Schmid MA, Ohteki T, Jarrossay D, Manz MG. Identification of clonogenic common Flt3+ M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow.
2007 Nature Immunology 8(11):1207-16.

Onai N, Obata-Onai A, Schmid MA, Manz MG.
Flt3 in regulation of type-I interferon producing and dendritic cell development.
2007 Ann N Y Acad Sci 1106:253-61.

Institute for Cell Biology & Immunology

University of Stuttgart
Germany

March – Dec 2005

Thesis (Diplom, Masters equivalent)

"PKD2 is recruited to the plasma membrane upon stimulation with PDGF in murine embryonic muscle cells"
Advisors: Dr Angelika Hausser, Prof Klaus Pfizenmaier

Publication

Eiseler T, Schmid MA, Topbas F, Pfizenmaier K, Hausser A. PKD is recruited to sites of actin remodelling at the leading edge and negatively regulates cell migration.
2007 FEBS Letters 581(22):4279-87.

Children's Cancer Institute Australia for Medical Research (CCIA)

University of NSW
Sydney, Australia

March – August 2003

Thesis (Bachelor equivalent)

"High-resolution cell division tracking of *in vitro* cultured childhood acute lymphoblastic leukemia xenograft cells"
Advisor: Dr Richard B Lock

Publications

Ong V, Liem NL, Schmid MA, Verrills NM, Papa RA, Marshall GM, Mackenzie KL, Kavallaris M, Lock RB. A role for altered microtubule polymer levels in vincristine resistance of childhood acute lymphoblastic leukemia xenografts.
2008 J Pharmacol Exp Ther 324(2):434-42.

Liem NL, Papa RA, Milross CG, Schmid MA, Tajbakhsh M, Choi S, Ramirez CD, Rice AM, Haber M, Norris MD, MacKenzie KL, Lock RB. Characterization of childhood acute lymphoblastic leukemia xenograft models for the preclinical evaluation of new therapies.
2004 Blood 103(10):3905-14.

Technical experience

- **Flow cytometric analysis and cell sorting**
  Eight color analysis, detection of cells with frequencies < 0,01%,
  intracellular stains, analysis of cell cycle proliferation
  Fluorescence activated cell sorting (FACS), four-way sorting
- **Mouse handling, experimentation and organ preparation**
  Injections & vaccination, bleeding animals, irradiation & generation of bone marrow chimera, clinical monitoring, cross breeding & screening, adoptive transfer of cells with congeneic marker expression, perfusions, magnetic bead selection (MACS, Miltenyi biotech, Germany)
- **Fluorescence microscopy, Laser scanning confocal microscopy (LSM)**
  of live or fixed tissues and cells
- **Gene expression analysis**
  RNA extraction, reverse transcription and real time-PCR (Taqman)
  of total organs, or single cells, as well small cell number (1000 cells)
- **Tissue culture**
  of cell lines and primary cells
- **Basic molecular biological & biochemical techniques**

Professional affiliations

- **American Society for Virology (ASV)**
  Toledo, USA
- **DC-THERA European network, Dendritic cells and Novel Therapies**
  Oxford, UK

Conferences & Workshops attended

- FACSAria Operator Practical Course, BD Biosciences
  2009 November, Erembodegem, Belgium
- American Society for Virology, 28th Annual Meeting (poster presentation)
  2009 July, UBC Vancouver, Canada
- Institute for Research in Biomedicine (IRB) student retreat 2008 (oral presentation)
  2008 November, Stels, Switzerland
- 10th international Symposium on Dendritic cells, DC 2008 (poster presentation)
  2008 October, Kobe, Japan
- EMBO Practical Course on Advanced Cytometry and Cell Sorting (oral presentation)
  2008 July, Berlin, Germany
- Alpine Meeting Molecular Basis of Disease of the ETH Zürich (oral presentation)
  2008 July, Richisau, Klöntal, Switzerland
- World Immune Regulation Meeting-II, WIRM II (oral presentation)
  2008 March, Davos, Switzerland
- Institute for Research in Biomedicine (IRB) student retreat 2007 (oral presentation)
  2007 May, Bigorio, Switzerland
- Immunology Summer School, EnII-Mugen (poster presentation)
  2007 April, Capo Caccia/Sardegna, Italy
- Graduate School DC-THERA, Dendritic Cell Network (oral presentation)
  2007 March, Celerina, Switzerland
- 9th International Conference on Dendritic Cells, DC 2006
  2006 September, Edinburgh, Scotland

Awards & Fellowships

May 2009	**American Society for Virology (ASV)**, Toledo, USA Travel Grant to join the 28th Annual ASV meeting 2009 Vancouver, Canada
March 2008	**Abcam Best Presentation Award Winner** World Immune Regulation Meeting-II, WIRM II Davos, Switzerland
July 2002	**German National Merit Foundation** "Studienstiftung des deutschen Volkes" Bonn, Germany
October 2001	**German Academic Exchange Service (DAAD)**, Bonn, Germany Scholarship for a one year study abroad program at the University of New South Wales, Sydney, Australia

Additional qualifications

Languages

- German native language
- English fluent
- Spanish intermediate
- Italian intermediate
- French intermediate

Employment experience

- Assisting scientist, practical lab work (part time, 2000 – 2002)
  University of Stuttgart, Germany
- Internship at "Sanofi-Aventis Pharma" (Oct – Dec 2004)
  Frankfurt am Main, Germany
- Community Service (July 1998 - July 1999)
  Clinics for Rehabilitation "Enzensberg", Hopfen, Germany

Teaching experience

- Tutor for undergraduate students (2000, 2001, and 2005)
  in Cell Biology, Inorganic Chemistry, and Mathematics
  University of Stuttgart, Germany
- Assistant in the practical course "Biophysics of the Cell" (2002)
  University of Stuttgart, Germany